DIFFERENTIATION AND DE-DIFFERENTIATION IN FRLA-209-15 CELLS AND THE POTENTIAL ROLE OF POLYAMINES

BY

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This dissertation is dedicated to Mom, Comfort, and Maxine.

Don't Quit

When things go wrong
  as they sometimes will,
When the road you are
  trudging seems all uphill,
When the funds are low,
  and the debts are high,
And you want to smile,
  but you have to sigh,
When care is pressing
  you down a bit--
Rest if you must,
  but don't you quit.

Success is failure
  turned inside out,
The silver tint of
  the clouds of doubt,
And you never can tell
  how close you are,
It may be near when
  it seems afar.
So, stick to the fight
  when you are hardest hit--
It's when things go wrong
  that you mustn't quit.

Author unknown

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS vi

LIST OF FIGURES ix

LIST OF TABLES xi

ABSTRACT xii

CHAPTER 1
- INTRODUCTION.. 1
  - Background 1
    - Properties and Postulated Functions of the Polyamines 2
    - Polyamines and Cell Growth 4
    - Polyamines and Differentiation 5
    - Polyamine Biosynthesis and the Cell Cycle 7
    - The effects of Inhibition of Polyamine Biosynthesis on Cellular Processes 8
    - Therapeutic Potential of Inhibitors 10
    - Newer Analogs 13
- Rationale.. 14
- Specific Aims.. 18

CHAPTER 2
- MATERIALS AND GENERAL METHODS.............................. 20
  - Materials 20
  - Methods 21
    - Cell Culture 21
    - Cell Counts and Viability 22
    - General Protocol 22
    - Protein Determination 23
    - Flow Cytometry 23
    - Polyamine Extraction and Detection 24
    - Preparation of Cell Extracts for Secreted Protein Studies 24
    - Two Dimensional SDS Polyacrylamide Gel Electrophoresis 25
    - Immunoblotting of Electrophoretically Transferred Protein 26
    - Receptor Binding Studies 27
    - MGBG Transport Studies 28
    - Whole Cell Cyclic Adenosine Monophosphate (cAMP) Assays 30
    - Neonatal Hepatocyte Isolation 31

CHAPTER 3

EFFECTS OF TEMPERATURE AND DFMO ON GROWTH AND LEVELS OF POLYAMINE IN FRLA-209-15 CELLS

EFFECTS OF TEMPERATURE AND DFMO ON GROWTH AND LEVELS OF POLYAMINE IN FRLA-209-15 CELLS 33
- Introduction 33
- Results 35
  - Effects of Temperature on Growth and Viability of FRLA-209-15 Cells 35
  - Effects of Temperature on Intracellular Polyamine Levels 36
  - Effects of DFMO on Growth and Viability of FRLA-209-15 Cells 37
  - Effects of DFMO Treatment on Polyamine Levels of FRLA-209-15 37
  - Flow Cytometric Analysis of Cells at the Permissive and Restrictive Temperatures 37
- Discussion 38

CHAPTER 4

CHARACTERIZATION OF PROTEIN SECRETION BY FRLA-209-15 CELLS AND ITS USE AS A SPECIFIC MARKER OF DIFFERENTIATION 50
- Introduction 50
- Results 52
  - Effects of Temperature on Total Secreted Proteins and Total Intracellular Protein 52
- Discussion 55

CHAPTER 5

THE β-ADRENERGIC RECEPTOR (βAR) AS A MARKER OF DIFFERENTIATION 69
- Introduction 69
- Results 72
  - Adrenergic Receptor Expression by FRLA-209-15 Cells 72
  - Course of Differentiation 73
  - Effects of Glucocorticoids (Betamethasone) on Differentiation of FRLA-209 Cells 74
  - Membrane Transport Changes in FRLA-209 Cells 74
  - Glucagon Stimulated Cyclase 75
- Discussion 75

CHAPTER 6

THE ROLE OF POLYAMINES IN DIFFERENTIATION AND DE-DIFFERENTIATION OF FRLA-209-15 CELLS 85
- Introduction 85
- Methods 88
- Results 88
  - Effects of Putrescine and Spermidine on Growth of FRLA-209-15 Cells 88
  - Effects of DFMO on the Phenotypic Expression of FRLA-209-15 Cells 89
- Discussion 90

CHAPTER 7

SUMMARY AND CONCLUSIONS.................................. 98

REFERENCES 109

BIOGRAPHICAL SKETCH 124

LIST OF FIGURES

Figure 3-1. Growth of transformed FRLA-209-15 cells at the permissive (33°C) and restrictive (40°C) temperatures.. 41

Figure 3-2. Cellular characteristics of FRLA-209-15 cells at the restrictive (40°C) and the permissive (33°) temperatures.. 43

Figure 3-3. Recovery of polyamine levels in FRLA-209-15 cells transferred from the restrictive (40°C) to the permissive temperature (33°C). 45

Figure 3-4. Effects of DFMO on FRLA-209-15 cells grown at the permissive temperature of 33°C................... 46

Figure 3-5. The effects of 5 mM DFMO on polyamine levels in FRLA-209-15 cells grown at 33°C............... 47

Figure 3-6. Effects of temperature and DFMO on the cell cycle phase distribution of FRLA-209-15 cells........... 49

Figure 4-1. Typical immunoblot analysis of proteins secreted by FRLA-209-15 at the permissive (33°C) and restrictive (40°C) temperatures......................... 61

Figure 4-2. Silver stained 2D-SDS-PAgels of proteins secreted by FRLA-209-15 cells at the restrictive (40°) and permissive (33°C) temperatures...................... 63

Figure 4-3. Immunoblot analysis of secreted proteins by FRLA-209-15 cells at the permissive (33°C) and restrictive (40°C) temperatures......................... 65

Figure 4-4. Immunostained 1D-SDS-PAGE showing the effects of temperature on proteins secreted by neonatal hepatocytes.................................... 67

Figure 5-1. A typical Scatchard plot of specific [^{125}I] CYP binding to whole cell homogenates from FRLA-209-15 cells grown at the permissive (33°C) and the restrictive (40°C) temperatures......................... 80

Figure 5-2. The accumulation of [^{14}C]MGBG into FRLA-209-15 cells grown at the permissive and restrictive temperatures.. 83

Figure 6-1. Effects of spermidine on the growth of FRLA-209-15 cells at 33°C.............................. 94

Figure 6-2. Immunostained 1D-SDS-PAgels showing the effects of DFMO on the transition of FRLA-209-15 cells from 40°C to 33°C..................................... 96

Figure 6-3. Laser densitometer quantitation of the anti-transferrin immunoreactive blot in fig 6-2 showing the effects of DFMO on de-differentiation....... 97

LIST OF TABLES

TABLE 3-1. Temperature effects on intracellular polyamine levels of FRLA-209-15 cells.................. 44

TABLE 4-1. Effects of temperature on total secreted and total intracellular proteins by FRLA-209-15 cells....... 59

TABLE 4-2. Effects of temperature on growth and viability of primary neonatal hepatocytes.............. 68

TABLE 5-1. Effects of differentiation on expression of βARs.. 81

TABLE 5-2. Effects of betamethasone on β-adrenergic receptor expression by FRLA-209-15 cells................ 82

TABLE 5-3. Effects of glucagon treatment on cAMP production by FRLA-209-15 cells........................ 84

TABLE 6-1. Effects of putrescine on growth and viability of FRLA-209-15 cells......................... 93

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FRLA-209-15 hepatocytes transformed with a temperature-sensitive SV40 mutant behave like differentiated cells at the growth restrictive temperature of 40°C and like transformed cells at the growth permissive temperature of 33°C. This study investigated the role of polyamines in the regulation of the transformed and differentiated phenotype in FRLA-209-15 cells. Experiments compared growth inhibition in FRLA-209-15 cells induced by temperature change to 40°C with growth inhibition produced by difluoromethylornithine (DFMO), an inhibitor of polyamine synthesis.

The secretion of the liver-specific proteins albumin and transferrin was characterized in the presence or absence of DFMO under the following conditions: 1) undifferentiated cells at 33°C; 2) differentiated cells at 40°C; 3) de-differentiated cells transferred from 40°C to 33°C. Secreted proteins were analyzed by 1-or 2- dimensional SDS-polyacrylamide gel electrophoresis followed by either silver staining or Western immunoblotting.

At 40°C, FRLA-209-15 cells showed immediate cessation of growth, a 100% increase in β-adrenergic receptor expression and a 50% decrease in the levels of putrescine and spermidine compared with cells at 33°C. In addition, at 40°C there was a 4-fold increase in total cellular protein, as well as a selective increase in the secretion of transferrin and albumin which is specific to differentiated FRLA-209-15 cells. Transfer of cells from 40°C back to 33°C resulted in the repletion of polyamines within 24 hours, followed by the re-emergence of the transformed phenotype. Moreover, protein secretion, expressed per million cells, decreased by 70-90% from levels observed at 40°C. In comparison, DFMO treatment at 33°C caused a gradual inhibition of growth and a > 90% depletion of both intracellular putrescine and spermidine, without an effect on protein secretion. Furthermore, addition of DFMO at the time of transfer from 40°C to 33°C slowed, but did not prevent the re-emergence of the transformed phenotype, suggesting that DFMO played a modulatory rather than a directive role in the phenotypic transition.

These findings suggest that although polyamines are important for proliferation in FRLA-209-15 cells, their role is modulatory as opposed to directive with respect to the expression of phenotype.

CHAPTER 1
INTRODUCTION

Background

The naturally occurring polyamines, spermidine and spermine, and the diamine, putrescine, are low molecular weight organic cations found in significant amounts in virtually all cells of higher eukaryotes (Pegg, 1982). When cells are depleted of their polyamines, by using highly specific inhibitors of synthesis, they cease to proliferate. Conversely, when polyamine-depleted cells are supplemented with polyamines, they resume a normal growth rate. In addition to their antiproliferative effect, inhibitors of polyamine synthesis induce terminal differentiation of certain tumors of embryonal origin and are antiparasitic. One of the inhibitors has been shown to cure patients with African sleeping sickness and to be effective against *Pneumocytis carinii*, an opportunistic protozoan infection in Acquired immune deficiency syndrome (AIDS) patients (Schecter *et al.*, 1987). Thus, it is evident that polyamines play an important role in cell growth and division. However, despite their ubiquity and extensive research efforts, the role of polyamines in cellular physiology, as well as their mechanism of action, remains vague (Pegg, 1988). Increased knowledge of the mechanisms that regulate intracellular polyamines promises to allow the design of novel antiparasitic, antiviral, and anticancer agents.

Properties and Postulated Functions of the Polyamines

The polybasic character of polyamines gives them a much higher affinity than Na^+, K^+, Mg^{2+}, or Ca^{2+}, for acidic macromolecules such as nucleic acids, certain proteins and phospholipids, and these interactions are very likely to influence the rate of various reactions. It has been suggested that polyamines contribute to neutralization of the negative charge on the DNA backbone. At a physiological pH, putrescine, spermidine and spermine are largely protonated and possess two, three, and four positive charges, respectively. The charge distribution in the spermine molecule allows it to bind strongly to two phosphate groups in each strand of the DNA double helix, spanning the minor or major groove, (Feuerstein and Marton, 1989) thus stabilizing the helix by binding its two strands together. Polyamines can cause major distortions of the usual B-form DNA, including transitions to both A- and Z-forms (Feuerstein *et al.*, 1991). These changes can have important consequences for DNA-protein interactions in the cell. The polyamines also stabilize other helical structures, such as stems and loops in rRNA and mRNA, and they stabilize tRNA conformation through binding to specific sites (Feuerstein and Marton, 1989). These interactions may be the basis for their stimulatory effects on DNA, RNA and protein synthesis. Consequently, in any given experiment, especially in *in vitro* studies, it is very difficult to know whether the effects observed have physiological relevance or are artifacts resulting from nonspecific polyamine-polyacid interactions (Tabor and Tabor, 1984). An excellent example comes from a study of the ionic requirements of the tRNA methyltransferases from rat liver (Morris, 1978). These enzymes are strongly activated by putrescine and spermidine at lower than physiological concentrations. However, even in the absence of polyamines two of the methyltransferases are fully active and the other 50% active. Moreover, neither tRNA or rRNA seems to be undermethylated in polyamine-limited mutants of *Escherichia coli* (Morris and Jorstad, 1973).

The importance of polyamines in cellular physiology both *in vitro* and *in vivo* has been demonstrated in mutant *Escherichia coli, Saccharomyces cerevisiae, Neurospora crassa, mouse lymphoma cells and chinese hamster ovary cells* (Holta and Pohjanpelto, 1982; Pohjanpelto *et al.*, 1981; Steglich *et al.*, 1982), which are defective in the biosynthetic pathway for the polyamines. A second line of evidence comes from experiments with difluoromethylornithine (DFMO), a reasonably specific, irreversible, mechanism-based ("suicide") inhibitor of ornithine decarboxylase (ODC). Studies such as these have firmly established the need for polyamines in the regulation of cellular proliferation (Heby, 1981; Tabor and Tabor, 1976) and differentiation

(Emmanuelsson *et al.*, 1978; Fozard *et al.*, 1980; Gazzit *et al.*, 1980; Takigawa *et al.*, 1980; Verma *et al.*, 1982), even though the reports on their role in differentiation have been conflicting.

Polyamines and Cell Growth

Stimulation of cell growth and division, both *in vitro* and *in vivo,* is associated with an increase in the rate of polyamine biosynthesis and concentration. Examples include initiation of growth in quiescent cell cultures by insulin or serum (Hogan *et al.*, 1974), after partial hepatectomy in rats (Janne *et al.*, 1968; Russel *et al.*, 1968), during embryonic development of a number of animal species (Caldarera *et al.*, 1970; Emmanuelsson *et al.*, 1978; Fozard *et al.*, 1980; Gazdar *et al.*, 1976), and after infection of cells with tumorigenic viruses (Gazdar *et al.*, 1976). Cell proliferation is preceded by increases in ODC activity. Inhibition of this initial rate limiting step in polyamine synthesis by α-DFMO arrests cell growth.

Difluoromethylornithine (DFMO) has been used to examine whether the increase in ODC activity and polyamine levels in preneoplastic cells is mandatory during chemical carcinogenesis. In one such study (Kingsnorth *et al.*, 1983) it was demonstrated that reduction of colonic polyamine levels after DFMO treatment of mice prevented proliferative changes induced by the carcinogen dimethylhydrazine and reduced the incidence of tumors. Other studies have shown that inhibition of polyamine metabolism may also restrict replication of some viruses in host cells (Pegg and McCann, 1982). DNA viruses, including vaccinia and human cytomegalovirus, cause an induction of ODC that may be necessary for viral replication in the host. With the use of methylglyoxal bis(guanyl-hydrazone) [MGBG], α-methylornithine, and later DFMO (Tyms *et al.*, 1982), it was shown that polyamine biosynthesis is an absolute requirement for the replication of these viruses. It has also been shown (Tyms *et al.*, 1982) that pretreatment with DFMO restricts replication of herpes simplex virus and Semliki forest virus.

Polyamines and Differentiation

Spermidine has been shown to be required for milk protein synthesis in cultured mouse mammary gland cells(Oka *et al.*, 1981). Further, the levels of this polyamine increase during differentiation of 3T3 cells (Bethel *et al.*, 1981). Sugiura *et al.* (1984) have demonstrated that spermidine is also required for differentiation of Friend murine erythroleukemia (MEL) cells and that intracellular spermidine levels correlate with the extent of differentiation. However, Heby (1983) reports that polyamines play a role in mouse embryonal carcinoma (F9 and PCC3) cell differentiation but, conversely, not in human promyelocytic leukemia (HL-60) cell differentiation. When HL-60 cells are induced to differentiate (Luk *et al.*, 1982) with phorbol ester (Huberman *et al.*, 1981), there is an associated increase in polyamine levels and ODC activity. The study, however, indicated that although ODC activity increases during both proliferation and differentiation, the increase is essential only for proliferation (Luk *et al.*, 1982). In contrast, treatment of human macrophage-granulocyte progenitor cells with DFMO results in a block of differentiation that can be reversed by the addition of exogenous putrescine (Verma *et al.*, 1982). The same authors (Sugiura *et al.*, 1984), who had previously demonstrated the involvement of spermidine in both the proliferation and differentiation of MEL cells, again report that spermidine is involved in HL-60 proliferation as well as in the induction of differentiation of HL-60 cells by dimethylsulfoxide, hexamethylene bisacetamide, butyric acid or retinoic acid. These are the very cell lines in which others have not found the involvement of polyamines in differentiation (Luk *et al.*, 1982). The more complete depletion of spermidine achieved by these authors (Suguira *et al.*, 1984) may explain this apparent discrepancy. Moreover, differentiation may require a critical number of cell replications (Suguira *et al.*, 1984) and slight differences in experimental conditions infuencing the involvement of polyamines in this process may contribute to the conflicting results. In some tumor cell systems, depletion of polyamines with DFMO prevents the development of the differentiated phenotype; in others it stimulates lines to differentiate (Chen *et al.*, 1983; Kapayaho *et al.*, 1985; Melino *et al.*, 1988; Oredsson *et al.*, 1985). This observation suggests that polyamines are involved in cell differentiation, even though the exact interrelationships between cellular proliferation and differentiation are unclear.

Polyamine Biosynthesis and the Cell Cycle

The question of polyamine function in regulation of cell cycle traversal has also been under investigation for a number of years (Heby *et al.*, 1977; Heby and Andersson, 1980; Oredsson *et al.*, 1983; Pegg and McCann, 1982; Prakash *et al.*, 1983; Rupniak *et al.*, 1980; Rupniak *et al.*, 1981; Seidenfeld *et al.*, 1981; Seidenfeld *et al.*, 1986; Sunkara *et al.*, 1981). Early studies used cell populations synchronized by mitotic shake off (Cress *et al.*, 1979; Fuller *et al.*, 1977; Heby *et al.*, 1976 and 1976b), while later studies used cells synchronized by excess thymidine (Hibami *et al.*, 1977). Both approaches involved correlation of changes in intracellular activities of polyamine biosynthetic enzymes and/or contents of the polyamines with progression through the cell cycle. Some investigators report two peaks of ODC activity and polyamine content as cells progress through the cycle (Heby *et al.*, 1976 and 1976b). The first peak occurs at or shortly before the onset of S phase and the second occurrs near the G_2-M border. Other workers who utilized excess thymidine synchronized cells report only single peaks of ODC activity in late G_1-early S (Cress *et al.*, 1979; Fuller *et al.*, 1977; Hibami *et al.*, 1977). These differences, however, may have resulted from the use of colcemid to maximize the yield of mitotic cells (Cress *et al.*, 1979; Fuller *et al.*, 1977) or the use of excess thymidine to induce synchrony (Hibami *et al.*, 1977). Nonetheless, these investigators agree that the regulation of polyamine content and biosynthetic activity during cell cycle progression implies a significant role for polyamines in cell cycle traversal. The question as to whether polyamines play a passive or a directive role in cell cycle traversal has not yet been resolved.

The Effects of Inhibition of Polyamine Biosynthesis on Cellular Processes

With the advent of specific inhibitors of polyamine biosynthesis, it became possible to partially deplete cells of their polyamine content and to investigate the consequences of such treatment on cell cycle phase distributions and rates of cell cycle traversal (Heby, 1983 Heby *et al.*, 1980 and 1981; Rupniak *et al.*, 1981). Treatment of cultured mammalian cells with DFMO results in a reversible inhibition of cellular proliferation. This is first observed when spermidine content is significantly reduced (Seidenfeld *et al.*, 1986). It remains a matter of controversy, however, if cells deficient in polyamine content are able to traverse all phases of the cell cycle or are blocked at one or more specific points (Heby and Andersson, 1980; Rupniak *et al.*, 1981). Several investigators have reported that normal rodent fibroblast cells are unable to transit from G_1 into S after treatment with inhibitors of polyamine biosynthesis, while transformed and/or tumor cell lines continue cell cycle traversal or become arrested in S phase under identical experimental conditions (Prakash *et al.*, 1983; Rupniak *et al.*, 1980; Sunkara *et al.*, 1981). When phase distributions are measured by premature chromosome condensation, it appears that some transformed or tumor cells accumulate in S phase in response to polyamine biosynthesis inhibitors (Prakash *et al.*, 1983; Sunkara *et al.*, 1981). These observations led to the proposition that such differences may be exploited to protect normal tissues from the effects of S-phase-specific cytotoxic agents, thus conferring a greater degree of antitumor selectivity to the use of such drugs. The rationale is that DFMO pretreatment would arrest normal cells in G_1 and thereby protect them from these S-phase agents. DFMO has been used in combination with S-phase-specific cytotoxic drugs such as cytosine arabinoside (Prakash *et al.*, 1983; Sunkara *et al.*, 1981) or hydroxyurea (Rupniak *et al.*, 1980). In such combinations synergistic cytotoxicities were reported. However, there have been recent reports indicating that DFMO treatment also protects 9L gliosarcoma cells from ara-C cytotoxicity (Oredsson *et al.*, 1983). Other workers do not observe an accumulation of cells in any one phase (Seidenfeld *et al.*, 1980), but report a lengthening of G_1-and S-phase transit times (Harada *et al.*, 1981). It is also of interest that polyamine-dependent (arginase-deficient) mutant Chinese hamster ovary cells respond to polyamine starvation with lengthening of, and the accumulation of cells in, the S and G_2 phases of the cycle (Anehus *et al.*, 1984).

Therapeutic Potential of Inhibitors

The association of increased levels of ODC and polyamines with rapid growth, differentiation, and cell cycle traversal has made the polyamine biosynthetic pathway an inviting target for agents that inhibit carcinogenesis, tumor growth (Pegg and McCann, 1982; Tabor and Tabor, 1984), and parasitic infections. Because DFMO and related drugs inhibit cell replication, there has been considerable interest in the potential therapeutic use of such compounds. In particular, a large number of studies in animals have shown the effectiveness of DFMO in inhibition of tumor growth, on interruption of pregnancy (Fozard et al., 1980; Lowkvist *et al.*, 1983; Reddy *et al.*, 1981), and in the treatment of protozoal infections (Gillet *et al.*, 1983; Hanson *et al.*, 1982; Sjoerdsma *et al.*, 1984). In *in vivo* studies in animals, the toxicity of DFMO was low, although reversible thrombocytopenia, leukopenia, and anemia are described (Luk *et al.*, 1983; Sjoerdsma *et al.*, 1984). In a number of clinical studies, patients with various types of tumors have been treated with DFMO, with DFMO plus MGBG, an inhibitor of S-adenosyl-methionine decarboxylase, or with these compounds in association with other chemotherapeutic agents. Despite the very promising animal and cell culture studies, the results of these clinical studies have been rather disappointing (Pegg and McCann, 1982). The reasons for the lack of activity of DFMO *in vivo* are unclear. It is possible that the depletion of polyamines in the tumors is not sufficient for a major response because of the availability of exogenous polyamines in the diet and compensatory increases in other enzymes in the biosynthetic pathway. There could also be limited accumulation of DFMO in cells, since no known transport mechanism other than simple diffusion has been described for its entry into cells. Another factor may be the difference between human tumors, which at the time of diagnosis frequently have already passed their most rapid phase of proliferation, and the animal models to which treatment is administered soon after tumor inoculation (Pegg and McCann, 1982).

In contrast, remarkable success has been achieved in the treatment of parasitic infections with DFMO either alone or in combination with other cytotoxic drugs like bleomycin (Fuller *et al.*, 1977). On the basis of this dramatic finding, Bacchi (1981) suggests that polyamine biosynthesis is a critical target for the development of further drugs against trypanosomatid flagellates. DFMO administered in the drinking water of mice infected with *Trypanosoma brucei brucei* is found to completely cure the mice within 3 days, a finding that led to a general upsurge of interest in the relationship between protozoan replication and polyamine biosynthesis (Bacchi *et al.*, 1980). Subsequent work has established that 1) this curative effect can be completely antagonized by administration of polyamines to infected animals during DFMO treatment, and 2) that DFMO does indeed inhibit *Trypanosoma* *brucei brucei* ODC and depletes putrescine and spermidine in the parasite (McCann *et al.*, 1981). Other experiments have demonstrated that ODC inhibition by DFMO will block replication of other African trypanosomes *in vivo* including *Trypanosoma brucei rhodesiense*, a cause of human sleeping sickness (McCann *et al.*, 1981). The unusual sensitivity of trypanosomes to DFMO has been attributed to the slow turnover rate of parasitic ODC which results in a much more prolonged inhibition (Heby and Persson, 1990). Treatment with DFMO may effectively reduce the polyamine level in the parasite, but not in its mammalian host where ODC turns over rapidly and where irreversibly inhibited ODC is continuously replaced by new active enzyme. Another possible contributor to the selective sensitivity of the parasite to DFMO is the reduced formation of trypanothione, a spermidine derivative, which acts as a free radical scavenger. The parasite *Eimeria tenella*, is an organism that causes coccidiosis in chickens. This parasite is completely prevented from dividing *in vivo* by DFMO, which cures the infection (McCann *et al.*, 1981). *In vitro* replication of the malaria parasite, *Plasmodium falciparum,* is also restricted by DFMO, which is shown to be mediated via inhibition of DNA synthesis (McCann *et al.*, 1981). The fact that polyamines administered simultaneously with the inhibitor reverse the inhibition and allow cell division to proceed in each of the above examples demonstrates the specificity of the DFMO effect.

Recently, in extension of these animal studies, DFMO has been used with success to treat *Pneumocystis carinii* pneumonia, an opportunistic protozoal infection in AIDS patients (Schechter *et al.*, 1987). In combination with acyclovir and ganciclovir DFMO has also been used in the treatment of cytomegalovirus infections in patients with AIDS (Tyms *et al.*, 1989).

Newer Analogs

Various attempts have been made to produce compounds that will effectively deplete cells of their polyamines in the hope of developing more potent antiproliferative agents. One such strategy has been the synthesis and use of bis(ethyl) derivatives of spermine and spermidine to suppress polyamine biosynthetic enzyme activity and, thereby, deplete intracellular polyamine pools (Bergeron *et al.*, 1989). These analogues behave similarly to the natural polyamines in suppressing the biosynthetic enzymes ODC and S-adenosyl-methionine-decarboxylase (Fogh *et al.*, 1975; Heby, 1983) and thereby deplete cells of polyamines. The depletion of polyamines is accompanied by a remarkable inhibition of cell growth which is thought to be due to the substitution of these analogs for polyamines in their ability to suppress enzyme synthesis but are incapable of supporting the growth promoting functions of natural polyamines. Recently it has been reported that these analogs, like natural polyamines, also stimulate the polyamine catabolic enzyme, spermine/spermidine acetyl transferase (Libby *et al.*, 1989). This observation raises the possibility that these analogs are themselves polyamines and that they support growth or inhibit growth depending on their concentration in the cell. This area needs further clarification, especially as these agents are now undergoing clinical trials as antiproliferative agents.

Rationale

Polyamines seem to be intimately associated with events in cell growth, or differentiation, and in the successful establishment of cell transformation induced either by chemical carcinogens or by viruses. In each case there appears to be an antecedent or associated increase in the activity of ODC and the biosynthesis of polyamines. Whether these changes are absolutely required for these processes has not been established and this question is the main emphasis of my dissertation.

The cell line FRLA-209-15 is a fetal rat liver cell that has been transformed with the temperature sensitive (*ts* A) mutant of simian virus (SV40) strain 209 (Chou, 1985). When grown at a permissive temperature (33°C), these cells exhibit several properties of a transformed cell line including: 1) decreased doubling time (rapid proliferation), 2) increased saturation density, 3) increased plating efficiency and 4) increased ability to overgrow a plate of normal fibroblast cells. At 33°C, these cells do not express the characteristics of a differentiated liver cell line, e.g. they do not synthesize and secrete the major serum proteins. Even if they do express low levels of such a function, expression is very low relative to what is observed when these cells are grown at 40°C. This difference is due to the fact that SV40 large T antigen (SV40LT) is only active at 33°C, but not at 40°C. T antigen binds and inactivates other nuclear and cellular proteins, like the retinoblastoma gene product (p105-Rb) and p53, which normally have a growth suppressor (antioncogene) functions. This inactivation of growth suppressor proteins ultimately leads to immortalization, enhanced proliferation, and loss of the differentiated cellular characteristics.

Interestingly, when FRLA-209-15 cells are shifted from the permissive temperature of 33°C to the restrictive temperature of 40°C, simian virus gene A product (SV40LT) is inactivated. SV40LT is responsible for induction and maintenance of the transformed phenotype in cells, and its inactivation causes them to exhibit characteristics consistent with those of differentiated fetal hepatocytes, for example, 1) prolongation or slowing of cell division, 2) decreased saturation density and plating efficiency, 3) inability to grow in soft agar, and 4) enhanced ability to synthesize and secrete serum proteins such as albumin, transferrin, and alpha fetoproteins (Chou, 1985). It has also been reported that transfer of these cells from the restrictive to the permissive temperature is associated with a two-fold increase in system A amino acid transport capability that is blocked in the presence of tunicamycin (Handlogten *et al.*, 1988). Whether the differentiation that occurs at the restrictive temperature occurs in a synchronous (i.e. in one phase of the cell cycle) or asynchronous fashion has not yet been determined.

Our preliminary data are interesting. We have shown that in the differentiated state there are accompanying surface molecular changes. The differentiated cells express about three times more β-adrenergic receptors (βARs) per mg protein than in the transformed state. This change provides an important and readily measurable end point for differentiation. We have also observed some interesting changes in the cellular polyamine levels as the cells proceed to differentiate; namely 1) an approximate 50% decrease in the intracellular levels of putrescine and spermidine and no significant change in spermine and 2) a six- to ten-fold decrease in the transport of exogenous MGBG and related natural or synthetic polyamines.

Whether these changes in polyamines play a directive or permissive role in the phenotypic transitions in FRLA-209 cells has not yet been established and is one of the questions I will address in this dissertation. It is not known whether polyamines are involved in the temperature-driven transformation of FRLA-209-40 cells. If they are, can the transition be abolished by preventing the accumulation of polyamine with inhibitors of ODC? It is hoped that answers to such questions may make the FRLA-209-15 cell line a useful and convenient model system with which to investigate these relationships.

One unique property of the FRLA-209 model system is that the differentiated cells can be induced to de-differentiate (i.e. re-assume the transformed phenotype) by changing the incubation temperature from 40°C back to 33°C. Conversely, we found that these cells can also be induced to arrest their growth at 33°C by decreasing polyamine levels with DFMO. A comparision between FRLA-209-15 cells growth-arrested at 40°C and cells induced to arrest at 33°C with DFMO will help our understanding of the role of polyamines in regulation of growth and differentiation of these virus-transformed cells.

Very little is known of the mechanisms of the transition from the differentiated to the de-differentiated state except that the cells regain their proliferative ability after a yet-to-be-defined point during incubation at the permissive temperature. It is of importance to determine when in the course of this transition the cells replete their polyamine levels. In addition, an examination of the effects of DFMO on polyamine repletion during the transition from differentiated to de-differentiated state may help explore whether polyamines play a directive or permissive role in these DNA virus transformed cells. For all of these reasons, these cells provide an interesting model system that can be used to probe the role of polyamines in virus-induced normal to neoplastic transitions.

Previous studies have compared tumor cells with primary cultures or with cells isolated from normal organs, and it has been very difficult to determine whether the observed changes are the cause or result of transformation. This system provides the advantage of looking at these parameters in the same cell line since it can be reversibly induced to grow, arrest, and differentiate or de-differentiate.

Specific Aims

We have confirmed that FRLA-209-15 cells shifted from the permissive (33°C) to the restrictive temperature of 40°C, stop proliferating, acquire the differentiated phenotype, and behave like hepatocytes. Another modality for slowing growth in FRLA-209-15 cells is DFMO treatment. Following the temperature-induced differentiation, there are apparent increases in cell size and the amount of βARs expressed per mg protein. Associated changes in the differentiated cell also include decreases in intracellular polyamine levels and MGBG transport capabilities. The significance of these changes remains to be established. A major objective of my research is to characterize the two modalities of growth inhibition, one with and the other without differentiation.

The overall hypothesis to be tested is that polyamines are essential for the differentiation and/or transformation of FRLA-209-15 cells. To address this hypothesis I intend to:

1 Characterize FRLA-209-15 cells with regard to their ability to alter the secretion of the hepatocyte-specific proteins albumin and transferrin during differentiation and transformation induced in response to temperature changes.

2 Examine whether polyamine depletion plays a directive or secondary role in the expression of the differentiated and transformed phenotype as measured by the differential secretion of the liver specific proteins transferrin and albumin.

3 Compare the characteristics of FRLA-209-15 cells growth inhibited by temperature with those inhibited by DFMO treatment with regard to growth, cell-cycle phase distribution, intracellular polyamine concentrations, and secretion of liver specific proteins albumin and transferrin.

CHAPTER 2
MATERIALS AND GENERAL METHODS

Materials

Blotting grade affinity purified goat antirabbit IgG horseradish peroxidase conjugate and reagents for sodium dodecyl sulfate (SDS)-polyacrylamide gels, electrophoresis grade, were obtained from Bio-Rad (Richmond, CA). Protein MW standards were purchased from Sigma Chemical Co. (St Louis, MO). Tissue culture supplies were purchased from Gibco (Grand Island, NY). *Staphylococcal aureas* protein A was iodinated by the chloramine-T technique to a specific activity of 6 μCi/μg and was provided by W. A. Dunn (Department of Anatomy and Cell Biology, University of Florida, Gainesville, FL). Rat albumin, rat transferrin, rabbit antiserum against rat albumin, and rabbit antiserum against rat transferrin were purchased from Cappel Laboratories Inc., Cochranville, PA. Hoefer SE700 multiple vertical slab gel units (Hoefer Scientific Instruments, San Francisco, CA) were used for 2-D electrophoresis. Nitrocellulose membranes (0.45 μm) for blotting were obtained from Schleicher and Schuell (Keene, NH).

Methods

Cell Culture

FRLA-209-33 cells (obtained from Dr. Janice Chou through Dr. Michael Kilberg) were maintained as monolayers at 33°C in alpha-minimum essential medium [α-MEM] supplemented with 2% heat inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.5 μg/ml) under 5% CO_2-95% air. Media were changed every other day and cultures were maintained as subconfluent monolayers by subculturing every third day. To divide cultures, monolayers were washed with prewarmed isotonic PBS (150 mM NaCl, 10 mM Sodium phosphate buffer, pH 7.4) then detached by transient exposure to an isotonic PBS solution containing 0.05% trypsin supplemented with 0.02% EDTA (to minimize clumping). The trypsin was removed and plates incubated for 1-5 minutes at 33°C. The rate and extent of detachment were monitored with an inverted microscope. When detachment was complete, the cells were suspended in a four-fold excess of complete media. Aliquots were taken and reseeded in fresh medium at starting concentration of 1.25×10^6 cells/25cm^2. Cells were kept in culture for a maximum of six weeks and then replaced with freshly thawed stock.

Cell Counts and Viability

Aliquots (100 µl) of cell suspension were diluted in 10 ml of Hematall diluent (Fisher Scientific, Orlando, FL) and cell number determined by electronic particle analysis with a Coulter counter (Model ZF, Coulter electronics, Hialeah, FL) and periodically confirmed with use of a haemocytometer (Reichert Scientific Instruments, Buffalo, NY). Cell viability was determined by trypan blue exclusion. Trypan blue dye (Eastman Kodak, Rochester, NY) was added to 100 µl aliquots of cells to a final concentration of 0.06%. The percentage of non viable (blue-stained) cells in a 10 µl aliquot was determined in a haemocytometer within 1 minute at room temperature.

General Protocol

FRLA-209-15 cells were initially grown at 33°C for 72 hours and then divided into three groups: 1) cells remaining at the permissive (33°C) temperature (transformed); 2) cells shifted to the restrictive (40°C) temperature (differentiated); and 3) cells shifted to the restrictive temperature for at least 48 hours and then moved back to the permissive temperature (de-differentiated). Based on preliminary studies, cells were incubated at 40°C for a minimum of 48 hours, after which time differentiation was complete based on albumin and transferrin secretion analysis.

Unless otherwise stated, transformed (33°C) cells, differentiated (40°C) cells, and de-differentiated (back to 33°C) cells refer, respectively, to the three groups defined above. One or more of these groups was treated with DFMO according to experimental design that will be described in the text.

Protein Determination

The protein content of either the cells or membranes was determined by the method of Lowry (Lowry *et al.*, 1951) or Bradford (Bradford, 1976) using bovine serum albumin as the standard.

Flow Cytometry

FRLA-209-40° cells, FRLA-209-33° cells in log phase, and DFMO treated FRLA-209-33° cells were subjected to flow cytometric analysis in accordance with the protocol of Braylan *et al.*(1984) with slight modifications. Briefly, the cells were lysed in 1 ml of nuclear staining solution (0.1% (w/v) sodium citrate, 0.03% (v/v) NP-40 detergent, RNase T1, 0.005% (w/v) propidium iodide) and filtered through a 44 µm pore-sized nylon mesh prior to the analysis. The separation of cells into phases was based on the differential DNA content of cells in G_1, S, and G_2/M respectively.

Polyamine Extraction and Detection

Cells (5 X 10^6) were collected by centrifugation for 5 minutes at 600 x g at room temperature. The supernatant fraction was removed and counted to assess cell loss. The cell pellet was resuspended in 1 ml of 50 mM phosphate buffer (pH 7.5), transferred into an eppendorf tube and cells were pelleted by centrifugation at 15000 x g for 1 minute. Again the supernatant was removed and analyzed for cell loss. The cell pellet was resuspended in 0.1 ml of ice cold millipore deionized water. Perchloric acid was added to a final concentration of 0.6 N (800 µl of 0.75 M stock); diaminohexane (internal standard) to give the equivalent of 2000 pmoles per million cells was added immediately (100 µl of 0.1 mM stock). The mixture was kept on ice for 30 minutes before the precipitate was removed by centrifugation at 15000 x g for 10 minutes. The supernatant fraction was stored at -20°C before polyamine quantification by HPLC (Bergeron *et al*, 1989).

Preparation of Cell Extracts for Secreted Protein Studies

Cultures seeded at 1 X 10^6 in 33 mm petri dishes were grown for 4 days at 33°C initially, with medium change every other day. At time zero (day 0 and a density of about 4 X 10^6 cells/plate) the cells were washed extensively with prewarmed (37°C) PBS, and the medium replaced with serum free medium.

The cultures were then divided into three groups: 1) cells remaining at 33°C; 2) cells transferred to 40°C for an additional 4 days of incubation; and 3) cells transferred to 40°C for 2 days and then transferred back to 33°C for 3 days. The medium was changed daily. Media and cells from duplicate plates of each group were collected and processed daily and stored at -20°C. Cells were lysed by the addition of 0.5 ml/plate of lysis buffer containing 5 mM K_2CO_3, 9.4 M urea, 2%(v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol (DTT) (Ogilvie *et al.*, 1989). Cell lysates were scraped from the culture dishes, sonicated at a setting of 3 for 10 seconds with a Braun-sonic 1510 probe sonicator, centrifuged at 15,000 x g for 30 minutes at 18°C, and the supernatants were collected. Samples of the medium and the supernatants from the cell lysates were extensively dialysed (M.W. 3,500 cut-off) in 10 mM Tris, pH 8.3, containing 0.02% (w/v) sodium azide at 4°C followed by 2 changes of deionized water. All samples were stored at -20°C.

Two Dimensional SDS Polyacrylamide Gel Electrophoresis

Proteins in samples of conditioned medium were analyzed by electrophoresis in the presesnce of sodium dodecyl sulfate (SDS) according to procedures described by Roberts *et al.*, 1984. Samples were lyophilized and resuspended in electrophoresis buffer, which contains 5 mM K_2CO_3 at pH 10.5, 9.3 M urea, 2% (v/v) Nonidet p-40, and 0.5% (w/v) DTT. Equal amounts of protein (100 μg) were loaded onto each gel, and proteins were resolved in the first dimension by isoelectric focusing in 4% (w/v) polyacrylamide tube gels [2.5 mm (internal diameter) x 11.5cm], containing 250 mM N,N'-diallytartardiamide, 8.0 M urea, 2% (v/v) Nonidet P-40, 0.5% (w/v) DTT, and 5.1% (v/v) ampholytes (pH 3-10, 5-7 and 9-11; 50:36:16, by volume respectively). Following isoelectric focusing, tube gels were equilibrated in 65 mM Tris-HCl containing 1% (v/v) β-mercaptoethanol and subjected to electrophoresis in the second dimension on 10% polyacrylamide gels in the presence of 1% SDS. The current settings, per gel, were 15 mA for 2 hours followed by 20 mA for 1 hour, and then 25 mA for 6 hours. Slab gels were either immunoblotted or fixed and silver-stained according to the procedure of Wray *et al.*, 1981.

Immunoblotting of Electrophoretically Transferred Proteins

Slab gels were equilibrated for 30 minutes in electrophoretic transfer buffer containing 0.025 M Tris-OH, 0.192 M glycine, 20% methanol, pH 8.3 (Towbin *et al.*, 1979). Proteins from gels were then transferred to nitrocellulose membranes at a constant current of 100 mA for 16 hours using the Bio-Rad Trans-Blot system (Bio-Rad Laboratories, Richmond, CA). Blots were rinsed with deionized H_20 and equilibrated in wash buffer (WB; 150 mM NaCl, 10 mM Tris-HCl, 0.2% (v/v) Triton X-100, 0.05% Na azide (w/v), pH 7.4).

Nitrocellulose membranes were blocked with 2% (w/v) gelatin in WB for 1 hour and rinsed 2x 5 minutes each in WB. Membranes were incubated overnight at 4°C with antiserum to rat albumin (rAlb) or rat transferrin (rTrf) diluted 1:2000 in WB containing 0.4% (w/v) bovine serum albumin (BSA). Membranes were washed four times for 5 minutes each with WB and incubated for 5 hours at 4°C with [^{125}I]-*staphylococcal aureas* protein A (1 x 10^6 cpm in 25 ml WB containing 0.4% BSA). Membranes were washed four times for 5 minutes each with WB, air dried and the immunoreactivity was visualized by exposure of the membranes to Kodak X-Omat AR film (Rochester, NY) for 1-2 days at -70°C.

Receptor Binding Studies

These studies were performed with transformed (FRLA-209-33) and differentiated (FRLA-209-40) cells.

Membrane preparations. The attached cells were rinsed once with ice-cold PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4). Cell monolayers rinsed with 10 ml of ice cold PBS were detached from the dishes with a rubber policeman and collected by centrifugation at 500 x g for 5 minutes. The cell pellet was then gently resuspended in either 0.5 ml or 2 ml of ice-cold 50 mM Tris-HCl buffer at pH 7.4, depending on whether they are for whole cell receptor binding assay or membrane studies, respectively. Cell samples for membrane studies were homogenized with a Tekmar SDT-100EN homogenizer (setting 2.5, 10 seconds), and centrifuged at 48,000 x g for 10 minutes in a Sorvall RC-5B centrifuge. The supernatants were discarded and the pellets washed twice in the same buffer, by centrifugation as described above. Finally, the pellets were resuspended in one volume of Tris buffer (as described above) for binding assays.

Binding assays. The α- and β-adrenergic receptors (αAR; βAR) were determined by specific [^{125}I]-2-[β-(4-Hydroxy-3-iodophenyl)-ethylaminomethyl]-tetralone (HEAT) and (−)-[^{125}I]-iodocyanopindolol (CYP) binding, respectively. Membrane or cell protein (0.05-0.10 mg) was incubated for 60 minutes at 36°C in a (50 mM Tris-HCl, 5 mM $MgCl_2$) buffer at pH7.4 containing 1) 6-100 pM [^{125}I]CYP with or without 1 μM alprenolol or 2) 6-100 pM [^{125}I]HEAT with or without 3.3 μM phentolamine for the measurement of βARs and αARs respectively. After incubation, each suspension was simultaneously diluted with 50 mM Tris-HCl buffer, filtered through Whatman GF/B glass fiber filters, and washed several times with Tris buffer using a Brandel cell harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD). The washed filters were placed in scintillation vials and counted in a gamma counter. Specific [^{125}I]CYP binding to the βAR was calculated as the difference between total binding in the absence of alprenolol and the non-specific binding in the presence of 1 μM alprenolol. The specific [^{125}I]HETE for the αAR was similarly calculated in the absence and presence of 3.3 μM phentolamine.

MGBG Transport Studies

The MGBG transport assay was based on the uptake of methylglyoxal-bis[^{14}C]guanylhydrazone ([^{14}C]MGBG) (specific activity 92.8 µCi/mg or 25.6 mCi/mmole, Amersham, Arlington Heights, IL). A stock solution of 1mM [^{14}C]MGBG was prepared aseptically and aliquots were added to the incubation medium to give the desired final drug concentration. Log phase (33°C) and differentiated (40°C) FRLA-209-15 cells were exposed to a range of concentrations of [^{14}C]MGBG for various times as described in the text. At the end of the incubation, the cells were washed 4 times with ice cold isotonic PBS containing 1 mM unlabelled MGBG. After the last wash, 0.5-1 ml of ice-cold PBS buffer supplemented with 0,02% (v/v) EDTA was added. The cells were gently scraped from the dishes, agitated thoroughly by trituration, and aliquots of 100 µl were taken and diluted in 10 ml Hematall for cell counting. Cell samples were transferred to scintillation vials for radioactive quantitation by liquid scintillation counting (Beckmann model LS 7000). The apparent intracellular concentration of [^{14}C]MGBG (in µM) was calculated assuming a volume of 0.568 µl per 10^6 cells. (A study comparing the size of FRLA-209-15 cells and L1210 cells relative to standard microspheres found them to be similar. Therefore, the volume previously determined for L1210 cells was used for my calculations.(Kanthawatana, 1989)). There were duplicate flasks for each drug concentration and the mean of three separate experiments was used for calculations. A few uptake studies, involving shorter time periods, were conducted in which the incubation temperatures were reversed (i.e. 33°C cells incubated at 40°C and vice versa) or in which cells were incubated at 37°C but the radioactive counts did not differ significantly.

Whole Cell Cyclic Adenosine Monophosphate (cAMP) Assays

FRLA-209-15 cells grown at 33°C for 4 days (one day before confluence) were used. Cells were washed twice with prewarmed (33°C) Hanks balanced salt solution (HBSS) and incubated in HBSS for 10 minutes to ensure temperature equilibration. Then 0.5 mM isobutylmethylxanthine (IBMX) with or without 0.1 µM of glucagon (Sigma, St Louis, MO), was added to the groups designated stimulated and basal, respectively. The cells were then incubated at 33°C for various times. At the end of the incubation the supernatant fraction was quickly aspirated and replaced with 1 ml of ice cold water. The cells were quickly scraped, pipetted into a microfuge tube, and placed immediately in a boiling water bath for 5 minutes before centrifugation in a microfuge at 13,000 x g for 5 minutes. The supernatant fractions were then used for the cAMP assay.

The cAMP content was determined by a competitive protein binding assay (Standifer et al., 1989). Briefly, incubations were performed in a total volume of 0.2 ml of 25 mM Tris-HCl buffer at pH 7.0, with 10 mM theophylline, 0.8 pmol of [^{3}H]cAMP, an aliquot of the assay supernatant, and 8 μg of bovine heart cAMP-dependent protein kinase for 60 minutes at 4°C. At the end of the incubation, 70 μl of a 50% (v/v) hydroxyapatite suspension was added to each tube followed by the addition of 4 ml of ice-cold 10 mM Tris-HCl buffer at pH 7.0. The suspensions were poured onto a Whatman GF/C glass filters under reduced pressure, washed with an additional 8 ml of ice-cold buffer and then placed in a scintillation vials with 1 ml of 0.3 N HCl. After the hydroxyapatite dissolved (about 20 minutes), 10 ml of liquiscint (National Diagnostics, Manville, NJ) was added and the radioactivity counted. The amount of cAMP present was calculated from a standard curve determined with known concentrations of unlabeled cAMP.

Neonatal Hepatocyte Isolation

Neonatal hepatocytes from 3 day old Sprague-Dawley rats were isolated by following the protocol described by Kraemer *et al.*, (1986) with slight modifications. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight). The liver was perfused *in situ* through the portal vein using modified calcium-free HBSS (HBSS, 0.35 g/L $NaHCO_3$, 100 mg/L penicillin/Streptomycin pH 7.4). The initial flow rate was 5 ml/minute for 5 minutes with the perfusate exiting via the severed inferior vena cava. After 5 minutes perfusion, the first solution was replaced by HBSS medium containing 0.05% (w/v) collagenase (Type CLS, Worthington Biochemical Corp., Freehold, NJ) and the perfusion continued for 4 minutes at 5 ml/minute.

The perfused liver was then removed, placed in a petri dish containing modified calcium-free HBSS and 5% fetal Bovine serum (FBS), and gently minced with sharp scissors to open the liver capsule. The minced liver suspension was filtered through a nylon mesh (60 µm pore size, Spectrum Medical Industries Inc., Los Angeles, CA). After filtration the cells were washed twice with calcium-free HBSS containing 5% FBS by centrifugation for 3 min at 50 x g. The harvested cells were enriched for hepatocytes by subjecting them to low-speed, iso-density percoll centrifugation in accordance with the "procedure B" described by Kraemer, *et al*., (1986). After washing the pelleted hepatocytes once, they were resuspended in RPMI-1640 containing 10% FBS. The cells were counted and then seeded in 33 mm petri dishes at initial concentrations of 7 x 10^6 cells at 37°C. Cell viability was determined by the trypan blue exclusion method.

CHAPTER 3

EFFECTS OF TEMPERATURE AND DFMO ON GROWTH AND LEVELS OF POLYAMINE IN FRLA-209-15 CELLS

Introduction

The naturally occurring polyamines, spermidine and spermine, and their diamine precursor, putrescine, are essential for cell growth, division, and differentiation (Marx *et al.*, 1987). Polyamines are ubiquitous in living tissues and are present in high concentrations in those tissues with high rates of synthesis of proteins, ribonucleic acids, and deoxyribonucleic acids (Fozard *et al.*, 1982). This correlation may be an indication of the fundamental importance of polyamines to the basic processes of cell growth and function. Polyamine biosynthesis is one of the molecular events that is stimulated during the G_1 phase of the cell cycle; polyamine synthesis has been implicated in the preparation of the cell for DNA replication (Heby *et al.*, 1981; Marx *et al.*, 1987). Polyamines also play an important regulatory function in the proliferation of malignant cells. A common property of many tumor promoters is their ability to induce increases in ODC activity (Moore *et al.*, 1982; Nakadate *et al.*, 1985; Pegg, 1988). The consequent increased production of putrescine appears to be critical for the induction of tumors. Tumor formation is prevented by the administration of substances which block the induction of ODC such as retinoids, inhibitors of arachidonic acid metabolism including indomethacin, or by direct inhibitors of ODC such as DFMO. DFMO is a specific, enzyme-activated, irreversible inhibitor of ODC, the rate-limiting enzyme in polyamine biosynthesis. ODC is responsible for the conversion of ornithine to putrescine, the first step in polyamine biosynthesis. Fluctuations in the level of intracellular polyamine have been observed in association with cell differentiation. DFMO inhibition of ODC activity causes rapid depletion of intracellular polyamine levels both *in vitro* and *in vivo* and thereby produces antiproliferative effects (Mamont *et al.*, 1978; Pegg and McCann, 1982). DFMO also blocks the retrovirus-induced transformation of mouse erythroid cell precursors (Klinken *et al.*, 1986). Depletion of putrescine with DFMO prevents the development of a differentiated phenotype in Friend erythroleukemia cells (Gazitt and Friend, 1980), chondrocytes (Takigawa *et al.*, 1980), L6 myoblasts (Ewton *et al.*, 1984), HL60 promyelocytes (Kufe *et al.*, 1984), and 3T3 fibroblasts (Erwin *et al.*, 1984). Moreover, in all cases the ability to differentiate is recovered on addition of polyamines to the culture medium (Melino *et al.*, 1988). In contrast polyamine depletion stimulates various tumor cell lines to differentiate (Chen *et al.*, 1983; Kapyaho *et al.*, 1985; Oredsson *et al.*, 1985). This observation suggests that polyamines are involved in cell differentiation, even though their role in the expression of the differentiated phenotype has yet to be clarified. This chapter describes experiments which explore the relationship between polyamines, cell-cycle transit, growth, and differentiation in FRLA-209-15 cells. The characteristics examined include 1) differences in growth between cells at the permissive and restrictive temperature in comparison with changes associated with DFMO induced inhibition; 2) differences in the intracelluar polyamine levels at the restrictive (40°C) and permissive (33°C) temperatures in comparison to the levels in DFMO inhibited cells; 3) flow cytometric analysis of the cell-cycle phase distribution of cells under these three conditions; 4) the effects of DFMO inhibition on the expression of the differentiated or transformed phenotype as measured by the secretion of the hepatocyte specific protein transferrin; and 5) a comparison of the ultra-structure of DFMO-treated cells and those growing at the permissive and restrictive temperatures.

Results

Effects of Temperature on Growth and Viability of FRLA-209-15 Cells

Figure 3-1 depicts the effects of temperature on growth of FRLA-209-15 cells. There was a dramatic slowing of growth at the restrictive temperature compared to those at the permissive temperature. This slowing of growth occurred within 24 hours of shifting to 40°C. The cells at 40°C appeared slightly larger than control, and numerous projections were evident (Figure 3-2) In addition, at 40°C, there was some degree of hypergranularity as well as the presence of small debris-like particles most of which excluded trypan blue. It was not clear whether these particles were buddings from the parent cells. Since the coulter counter did not distinquish between the debris-like particles and the cells, a haemocytometer was used for accurate cell counts. In addition, cell viability deteriorated to about 50% that of control after 6 days of growth at the restrictive temperature. For these reasons, further studies were routinely limited to 4 days at the restrictive temperature.

Effects of Temperature on Intracellular Polyamine Levels

The effects of temperature on intracellular polyamine levels was determined as described in Chapter 2. Table 3-1 illustrates the effects of temperature on intracellular polyamine levels. At 40°C, FRLA-209-15 cells showed a significant decrease (>50%) in the levels of putrescine and spermidine compared with cells at 33°C. The spermine levels were not significantly altered.

Transfer back to 33°C from 40°C resulted in a repletion of polyamines within 24 hours (Figure 3-3). In fact spermidine and spermine levels rose significantly above those of control ($p<0.01$), remained at these levels for 48 hours, but returned to control levels by 72 hours.

Effects of DFMO on Growth and Viability of FRLA-209-15 Cells

The effects of DFMO on growth and viabilty of FRLA-209-15 cells at the permissive temperature of 33°C is shown in Figure 3-4. Whereas treatment of cells with 1 μm-1 mM of DFMO for 6 days did not significantly affect growth and viability (data not shown), growth was noticeably inhibited after 4 days of treatment with 3-5 mM DFMO. However, there was no difference in viability between the treated and untreated cells. These results suggest that DFMO's effects were cytostatic as opposed to cytotoxic in nature.

Effects of DFMO Treatment on Polyamine Levels of FRLA-209-15

Figure 3-5 depicts the intracellular polyamine levels of DFMO treated cells. During the first three days of treatment spermidine levels gradually declined to approximately 50% of control whereas spermine levels rose to about 180%. Thereafter there was a time dependent decline in both spermidine and spermine. As indicated by the arrow, growth inhibition correlated well in time (after 4 days of treatment) with the decline in spermidine levels.

Flow Cytometric Analysis of Cells at the Permissive and Restrictive Temperatures

To investigate whether the depletion of polyamine levels and the growth inhibition accompanying either DFMO treatment or transfer of FRLA-209-15 cells to 40°C was associated with arrest in a particular phase of the cell cycle, the phase distribution of these cells was analyzed by flow cytometry. Figures 3-6 depicts typical flow cytometric analyses of FRLA-

209 cells at the permissive and the restrictive temperatures. Transfer to 40°C was accompanied by a block at the G_1-S border and a prolongation of the S and G_2 phases. As depicted in the lower panel, DFMO treated FRLA-209 cells accummulated in G_1.

Discussion

The data support the report of Chou *et al.* (1985) and others (Handlogten *et al.*, 1988) that FRLA-209-15 cells are temperature sensitive with respect to growth. The cells grow rapidly at the growth permissive temperature of 33°C, but at the restrictive temperature of 40°C growth is slow or virtually absent (Figure 3-1). We have also shown that the growth arrest occurs in association with a cessation in the synthesis of new DNA (Figure 3-7).

The transforming region of the DNA tumor virus, SV40, encodes functional antigens (T antigens) at 33°C which confer the transformed phenotype on these cells. The exact mechanism of transformation is unclear, but it is believed that the T antigen, a largely nuclear phosphoprotein of 708 amino acids, forms a complex with a number of cellular proteins. Such proteins include the retinoblastoma gene product (p105Rb) and P53 (Dyson *et al.*, 1989; Mitchell *et al.*, 1987; Whyte *et al.*, 1988), which normally provide a growth suppressor function (antioncogenes) in cells. The removal of these factors is followed by uncontrolled growth which is characteristic of transformed and neoplastic cells (DeCaprio *et al.*, 1988). At 40°C, the T antigen in temperature sensitive FRLA-209-15 is inactivated, the cells assume their original hepatocyte characteristics, and proliferation is curtailed (Figure 3-1). When cells at the restrictive temperature are moved to the permissive temperature the process is reversed.

Furthermore, intracellular polyamine levels of FRLA-209-15 cells, like growth, are temperature dependent. Movement of cells to the restrictive temperature, was associated with significant decreases of putrescine and spermidine (Table 3-1). The reversibility of the process was demonstrated by the repletion of polyamine levels within 24 hours after transfer back to 33°C from 40°C. Although there were differences in the absolute levels of polyamines from experiment to experiment, the relationship in ratios within experimental groups was the same. At times the putrescine peaks were low and difficult to measure, but the levels of spermidine and spermine were measured with consistency.

DFMO decreases the proliferation of a variety of cells either *in vivo* or *in vitro*. It has also been shown by others (Klinken *et al.*, 1986; Suguira *et al.*, 1984) that DFMO is able to severely restrict replication and differentiation in a number of cells. I have compared the effects of DFMO and temperature on FRLA-209-15 cells. The data show that like temperature, DFMO causes growth inhibition of FRLA-209-15 cells. However, whereas inhibiton due to temperature is immediate, inhibition due to DFMO is gradual with a lag period of 4 days. Moreover, flow cytometry studies suggest that the temperature-induced growth arrest occurrs at both G_1-S interface and G_2/M, whereas the growth arrest due to DFMO occurred at G_1 (Figures 3-7 and 3-8).

My results have also demonstrated that the inhibition of FRLA-209-15 cell growth induced by both temperature and DFMO is associated with a depletion of intracellular polyamines, the extent of which is greater in the case of DFMO. Additionally, the depletion of spermidine correlates well with the onset of growth inhibition in the case of DFMO. As will be described in Chapter 4, another important difference between the two modalities of growth inhibition is the fact that whereas temperature induces the expression of the differentiated phenotype, as indicated by enhanced secretion of liver proteins (Figure 4-1), DFMO does not. These data suggest that even though polyamine changes occur in the temperature-induced differentiation of FRLA-209-15 cells, these changes may not play a directive role in the phenotype of protein synthesis.

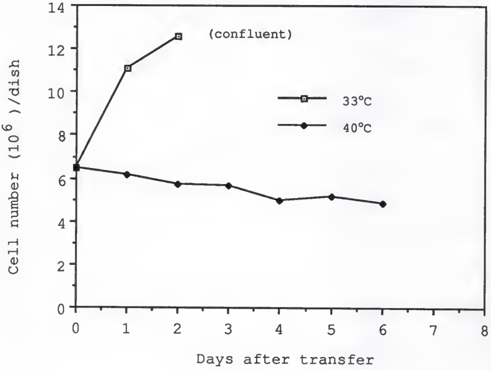
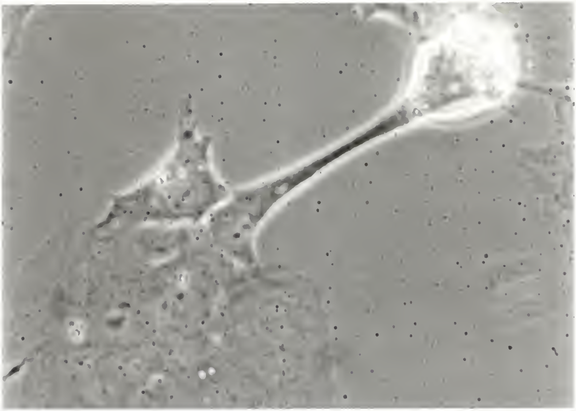


Figure 3-1. Growth of transformed FRLA-209-15 cells at the permissive (33°C) and restrictive (40°C) temperatures.

Cells were grown as monolayers in alpha-Minimum essential medium (α-MEM-2) supplemented with 2% heat-inactivated fetal bovine serum; the medium was changed every other day. Cells were initially grown at 33°C for 3 days (day 0) then divided into the two respective temperatures of 33°C and 40°C. Cells were counted every day with a haemocytometer and viability determined by the trypan blue exclusion method as described in the "Methods" section.

Figure 3-2. Cellular characteristics of FRLA-209-15 cells at the restrictive (40°C) and permissive (33°C) temperatures.

Cells were grown on cover slips for 3 days then divided into two groups. One group was moved to 40°C while the other remained at 33°C for 2 additional days. At the end of the incubation, cells were washed with serum-free α-MEM, stained with Rhodamine, and examined with a Zeiss fluorescent microscope. (Top panel) Cells at 40°C; (Bottom panel) Cells at 33°C.



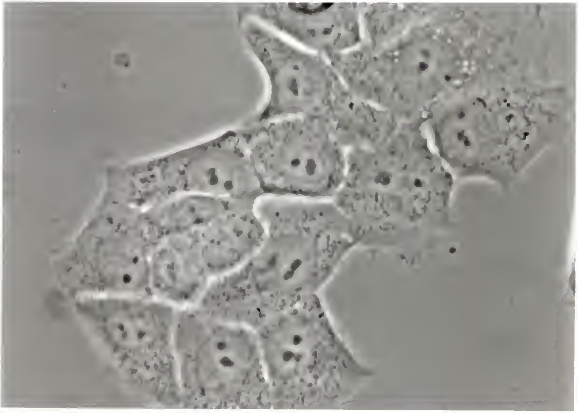


TABLE 3-1

Temperature effects on intracellular polyamine levels of FRLA-209-15 cells

Cell Incubation Temperature	Putrescine	Spermidine	Spermine
	pmoles/10^6 cells		
33°C	345 ± 27	3061 ± 216	1540 ± 149
40°C	128 ± 11	1113 ± 28	1223 ± 120
	(-62.9%)a	(-63.7%)a	(-20.6%)

Results are expressed as Mean ± SE

[a] significant at $p<0.05$

FRLA-209-15 cells were grown initially at 33°C for 3 days and then split into two groups, namely 33°C and 40°C. Cells were incubated at these temperatures for an additional 48 hours before processing samples for polyamine extraction as described in the "Methods" section. Each value represents the mean ± SE of triplicate determinations. Polyamine levels are expressed as pmoles/million cells.

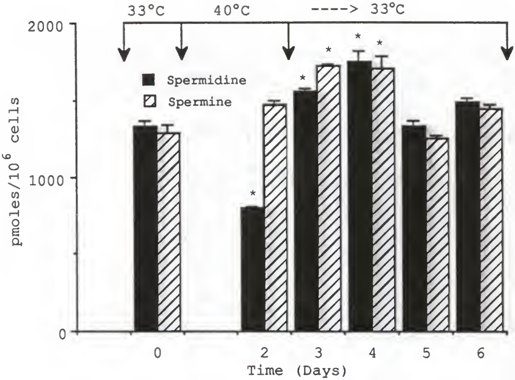


Figure 3-3. Recovery of polyamine levels in FRLA-209-15 cells transferred from the restrictive (40°C) to the permissive temperature (33°C).

Cells were initially grown at 33°C for 3 days (represented as day 0) then divided into the control (33°), the differentiated (40°C), and the de-differentiated (back to 33°C from 40°C) groups. The control cells (33°C) were processed for determination of polyamine levels, and the remaining cells were moved to 40°C for an additional 48 hours. Samples of the cells at 40°C (differentiated group) were processed for polyamine extraction, and the remaining cells (de-differentiated group) moved back to 33°C for 4 days. The polyamine levels were then determined at 24 hour intervals. Polyamines were measured as described in the "Methods" section. The results are expressed as mean ± SE of 2 determinations assayed in quadruplicate. * indicates significance at $p<.001$ using ANOVA.

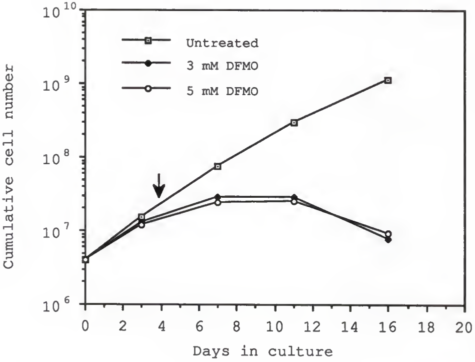


Figure 3-4. Effect of DFMO on FRLA-209-15 cells grown at the permissive temperature of 33°C.

Cells were grown in α-MEM-2, and the medium was changed every other day. DFMO was added 24 hours after initial seeding (day 0) and maintained in the medium at each medium change or passage. Cells were counted at the various times with a Coulter counter (model ZF) and cross checked with a haemocytometer. Viability was determined by the trypan blue exclusion as described in "Methods". Each data point represents the mean of 2 separate experiments, each in duplicate.

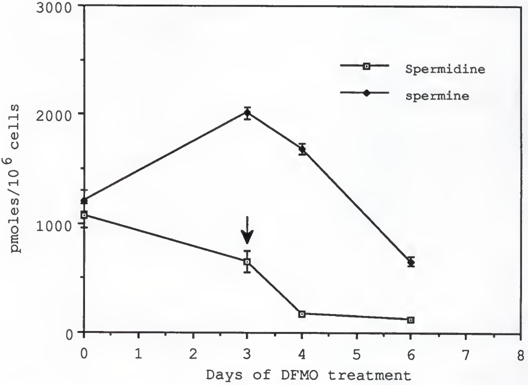
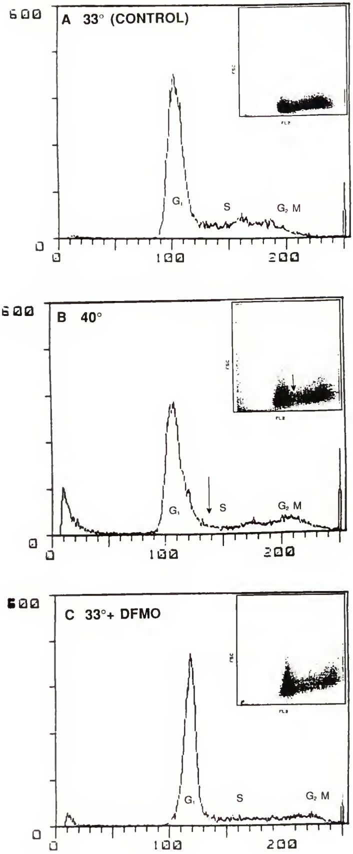


Figure 3-5. The effect of 5 mM DFMO on polyamine levels in FRLA-209-15 cells grown at 33°C.

Cells were grown and processed for polyamine determinations as described in the "Methods" section. DFMO was added 24 hours after the initial seeding and maintained at each medium change. The values are expressed as pmole/million cells ± SE and each represents the mean of 2 experiments each done in quadruplicate. The arrow marks the time at which growth inhibition was detected.

Figure 3-6. Effects of temperature and DFMO on the cell cycle phase distribution of FRLA-209-15 cells.

Two groups of cells were initially grown at 33°C. One group was exposed to 5 mM DFMO 24 hours after seeding for 8 days while the other group, after 3 days of growth at 33°C, was split between 40°C and 33°C for an additional 2 days. Cells were then processed for analyses as described in the "Methods" section.(Top) Cells at 33°C; (Middle) Cells at 40°C; (Bottom) 5 mM DFMO treated cells. (Inset) Data are presented in a blot form.


600
A 33° (CONTROL)
FSC
FL2
G1
S
G2 M
0
0
100
200
600
B 40°
FSC
FL2
G1
S
G2 M
0
0
100
200
600
C 33°+ DFMO
FSC
FL2
G1
S
G2 M
0
0
100
200

CHAPTER 4

CHARACTERIZATION OF PROTEIN SECRETION BY FRLA-209-15 CELLS AND ITS USE AS A SPECIFIC MARKER OF DIFFERENTIATION

Introduction

The usefulness of conditionally lethal mutants for studying structure and function in biological materials has been amply demonstrated. In the past 20 years many cell cycle-specific mutants have been isolated and partially characterized, both in yeast and in animal cells (Basilico, 1977; Hartwell *et al.*, 1976; Travali *et al.*, 1990).

Cells that retain differentiated tissue-specific functions have been established by transforming normal differentiated cells with temperature sensitive (ts) mutants of RNA or DNA viruses that are temperature sensitive for maintenance of transformation (Boettiger *et al.* 1977; Chou, 1978; Pacifici *et al.* 1977). Based on a variety of criteria, such transformed cells behave like tumor cells at the permissive temperature, but mimic the normal nontransformed tissues at the nonpermissive temperatures.

Chicken embryo retinal melanoblasts (Boettiger *et al.*, 1977) and chicken chondroblasts (Pacifici *et al.*, 1977) that retain differentiated functions have been established by the transformation of normal melanoblasts and chondroblasts with ts mutants of *Rous sarcoma* virus. Human placental trophoblasts that retain differentiated placental functions have been established by the transformation of normal placental cells with ts mutants of SV40 (Chou, 1978). The transformed phenotype and the differentiated phenotype of all three types of cells are temperature-dependent. At permissive temperatures, the transformed melanoblasts cease melanin synthesis and degrade their melanosomes; transformed chondroblasts stop synthesizing sulfated proteoglycans; and transformed human placental cells synthesize low levels of human chorionic gonadotropin (hCG) and its alpha subunit (hCGα) in addition to having low alkaline phosphatase activities. At nonpermissive temperatures, transformed melanoblasts actively synthesize melanin; transformed chondroblasts reinitiate the synthesis of sulfated proteoglycans; and transformed placental cells again synthesize high levels of hCG as well as hCGα, and they have high alkaline phosphatase activities.

Chou *et al.* (1980) established the (FRLA-209-15) cell line by the transformation of normal fetal liver cells with the SV40 *tsA209* virus. FRLA-209-15 cells are temperature dependent for the regulation of albumin, transferrin, and α-fetoprotein synthesis. Albumin and transferrin are two characteristic products of adult, vertebrate liver and the study of the control of the synthesis and secretion of these proteins may reveal the regulatory mechanisms involved in development and terminal differentiation. Monitoring the levels of secretion of albumin and transferrin in relation to temperature changes may serve as an important marker of differentiation, which could be used to explore the role that polyamines play in the regulation of the transformed and differentiated phenotype in FRLA-209-15 cells.

Results

Effects of Temperature on Total Secreted Proteins and Total Intracellular Protein

The effects of temperature on the synthesis and secretion of proteins by FRLA-209-15 cells grown as described in earlier chapters were studied. Total secreted and intracellular proteins were quantitated using the Bradford assay, and the results expressed per million cells. Table 4-1 shows the results of a typical experiment. At 40°C there was a 4-fold increase in total cellular and total secreted proteins per million cells per 24 hours. Transfer of cells from 40°C to 33°C was associated with a return of protein levels within 24 hours to levels observed at 33°C, a finding that demonstrates the reversibility of the temperature-effects.

To confirm that the FRLA-209-15 cells were secreting the hepatocyte-specific serum proteins, the effects of temperature on the secretion of albumin and transferrin was examined at the nonpermissive temperature using 1-dimensional SDS-PAGE followed by Western immunoblotting. Dialysed culture medium from 2 x 10^6 cells was applied per lane. Figure 4-1 (middle panel) shows the induction of albumin secretion at the nonpermissive temperature of 40°C. FRLA-209-15 cells produced low levels of albumin at 33°C. When FRLA-209-15 cells were shifted from 33°C to 40°C, albumin secretion greatly increased. Maximal increase in albumin secretion appeared 48 hours after transfer to 40°C, suggesting that the expression of the differentiated phenotype, as measured by protein secretion, was complete by 48 hours. The increase in albumin secretion at 40°C was reversible. When cultures that had been at 40°C for 48 hours were shifted back to 33°C a decrease in albumin secretion was seen (Figure 4-1; middle panel). Albumin levels returned to the control levels at 33°C, 48 hours after shifting from 40°C to 33°C. This delay suggests that it takes about 2 days for the transition from the differentiated to the transformed phenotype to be completed.

Figure 4-1 (top panel) shows the induction of transferrin secretion in FRLA-209-15 cells at the nonpermissive temperature. At 33°C, the secretion of the liver specific protein transferrin was low. The shift from 33°C to the nonpermissive temperature of 40°C was associated with greatly increased secretion of transferrin which peaked by 48 hours. Transferrin secretion at 40°C remained high until the cells were transferred back to 33°C. When cultures that had been incubated at 40°C for 2 or more days were shifted back to 33°C, transferrin production decreased, demonstrating again the reversibility of this phenomenon.

The induction of the synthesis of both intracellular and secreted proteins by FRLA-209-15 cells at 40°C was clearly apparent in table 4-1 and Figure 4-1. This prompted further examination of total secreted proteins in order to determine whether the general increase in protein synthesis is accompanied by a selective expression of the liver specific proteins albumin or transferrin. Equal amounts (100 μg) of conditioned media from FRLA-209-15 cells grown at the permissive (33°C) and nonpermissive (40°C) temperatures were analysed by two-dimensional SDS-PAGE followed by either silver staining or western immunoblotting. Figure 4-2 (top and bottom panels) shows the complete profile of proteins secreted by FRLA-209-15 cells at 40°C and 33°C, respectively. Although cells at 40°C secreted substantially more proteins, the 2D profile of the proteins was comparable for cells at 33°C and 40°C.

Analysis of Western immunoblotted 2D-SDS-PAgels using rabbit anti-rat serum albumin (α-rSA) antibody showed that at 40°C there was a substantial increase in the amount of immunoreactive albumin present in culture media which exhibited a molecular weight in agreement with that published in the literature (Figure 4-3). The bottom panel shows low levels of immunoreactive protein in culture medium from FRLA-209-15 cells at 33°C.

The effects of temperature on protein secretion by primary neonatal hepatocytes was studied to determine whether the increase in proteins observed in FRLA-209-15 cells at

40°C (Table 4-1) was specific to SV40 transformed fetal hepatocytes. Conditioned media from 2 x 10^6 primary neonatal hepatocytes grown under identical conditions as described in the "Methods" section were analyzed by one-dimensional-SDS-PAGE. Figure 4-4 shows that in contrast to the observations with FRLA-209-15 cells, exposure of primary cultures of neonatal hepatocytes to 40°C resulted in a decrease in secreted transferrin compared to those of cells at 33°C and 37°C. These data suggest that the temperature-induced increased secretion of transferrin by FRLA-209-15 cells was specific to FRLA-209-15. It must also be noted that the level of secretion of the serum protein transferrin by the neonatal hepatocytes was much greater than that observed in FRLA-209-15 cells based on the observation that exposure to X-ray film had to be limited to 3-6 hours as opposed to the usual 24-48 hours in the case of FRLA-209-15 cells.

Discussion

The data presented here support the finding that FRLA-209-15 cells are temperature sensitive with respect to the expression of the transformed or differentiated phenotype, and that the phenomenon is reversible. When transformed cells are shifted from the permissive (33°C) to the restrictive temperature (40°C), the cells acquire the differentiated phenotype and behave like hepatocytes. In addition to curtailed cell proliferation, the synthesis and secretion of the hepatocyte specific proteins, albumin and transferrin, increases at the restrictive temperature. These data have demonstrated that in addition to secreted proteins, at 40°C, there is a general increase in the expression of total cellular protein (Table 4-1). This study used Western immunoblotting as opposed to the radioimmunoassay or immunoprecipitation techniques used by Chou *et al.*(1981).

The data suggest that, consistent with the observations summarized in Table 4-1, cells at the restrictive temperature (40°C) have a greatly increased capacity for secretion of total proteins as well as the liver-specific proteins albumin and transferrin. This specific increase in proteins is observed when samples are normalized either on a per "million cells" (Figure 4-1) or a "per mg protein" basis (Figures 4-2 and 4-3).

The appearance and disappearance of the liver specific proteins, albumin and transferrin, in response to temperature (Figure 4-1) affords the opportunity to monitor the course and kinetics of differentiation and de-differentiation. It was concluded that at 40°C, the process of differentiation is virtually immediate in onset and is complete by 48 hours. When differentiated cells are shifted to 33°C, the return to the transformed state is immediate and is complete by 72 hours. It should be emphasized that this change correlates with the return of polyamine levels to the control levels as described in the previous chapter. The overall results are in agreement with the patterns observed by Chou *et al.* (1981 and 1985).

Lowered albumin levels have been observed during acclimatization to heat (Rothschild *et al.*, 1988). Moreover, experimental studies in rats during the first 2 weeks of heat exposure found that albumin synthesis decreases by 60% (Oratz *et al.*, 1967). Exposure to cold does not alter albumin synthesis (Coetzee *et al.*, 1982). Our studies with primary neonatal hepatocytes paralleled this observation. A shift from 33°C to 40°C or 37°C to 40°C is associated with a decrease in secreted transferrin. However, a shift from 37°C to 33°C is associated with no change or a slight increase in secreted transferrin (Figure 4-4). In contrast, a shift of FRLA-209-15 cells from 33°C to 40°C, is associated with an increase in secreted transferrin. These observations suggest that the temperature-induced increased secretion of transferrin by FRLA-209-15 cells is a specific phenomenon, and not a general response to temperature increase. Overall, the quantity of transferrin secreted by the primary hepatocytes at the two temperatures is greater than the amount secreted by the FRLA-209-15 cells. Possibly, the process of viral transformation affects the amount of specific as well as general proteins that can be secreted. Another potential explanation is that some protein secretory capabilities of FRLA-209-15 cells have been gradually lost as a result of prolonged culturing. Chou *et al.*(1985) have reported the loss of all or some of the protein secreting characteristics of FRLA-209-15 cells after 3 months of growth in culture. We have also observed a decrease in the amount βAR as well as a loss of differential expression of βAR at both temperatures following prolonged culturing. It is also possible that the primary neonatal hepatocytes have gone through the developmental switch from the secretion of predominantly α-fetoprotein to that of albumin, transferrin, AGP, and TAT that occurs at the time of birth. On the contrary, FRLA-209-15 cells are induced by temperature to differentiate into fetal hepatocytes (Chou, 1988) and there is an increase in secreted α-fetoprotein, albumin, transferrin.

TABLE 4-1

Effect of temperature on total secreted and total intracellular proteins by FRLA-209-15 cells

Cell Incubation Temperature	Cells/dish $x10^6$	Media µg protein/ 10^6 cells	Cell Lysate µg protein/ 10^6 cells
33°C	6.1	21 ± 2	220 ± 19
$40°C_{2d}$	4.3	122 ± 8 *	422 ± 31 *
$40°C_{4d}$	2.8	140 ± 13 *	678 ± 40 *
$-->33°C_{1d}$	6.2	20 ± 1.8	259 ± 12
$-->33°C_{2d}$	6.9	29 ± 3	275 ± 23

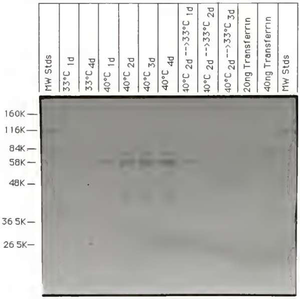
--> indicates cells grown at 40°C for two days then moved to 33°C for the stated times.

* Significant with respect to control (33°C) at $p<0.01$

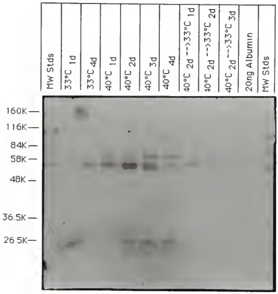
FRLA-209-15 cells initially grown at 33°C for 3 days were divided into three goups, control (33°C); differentiated (40°C); and de-diferentiated (from 40°C back to 33°C). The control cells were left at 33°C for the stated time, and the remaining groups shifted to 40°C. After 48 hours incubation at 40°C, the de-differentiated group was transferred back to 33°C for the stated times. At each time point conditioned media and cell lysate samples were prepared as described in the "Methods" section. Total secreted proteins and cell lysate proteins were measured using the Bradford method. The results are expressed as mean ± SE of 3 determinations, each of which was assayed in duplicate.

Figure 4-1. Typical immunoblot analysis of proteins secreted by FRLA-209-15 at the permissive (33°C) and restrictive (40°C) temperatures.

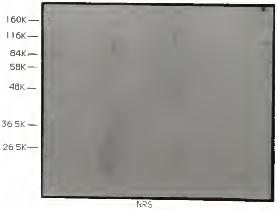
FRLA-209-15 cells initially grown in complete α-MEM-2 for 3 days were extensively washed with PBS; the cells were then incubated in serum free α-MEM-0 medium. The cells were then divided into 3 groups, 1) 33°C cells; 2) 40°C cells; and 3) cells transferred from 40°C to 33°C. The cells in all three groups were incubated in serum free media at their respective temperatures for the indicated times. The conditioned medium was then collected for analysis by electrophoresis on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunostained with the appropriate rabbit anti-rat serum primary antibody, using ^{125}I labelled protein A for immunodetection. Protein from 2×10^6 cells was loaded per lane. (Top panel) Immunostained with anti-rat serum albumin antibody (α-rSA); (Middle panel) Immunostained with anti-rat transferrin antibody (α-Trf); (Bottom panel) Immunostained with pre-immune serum (NRS).



ANTI-TRANSFERRIN



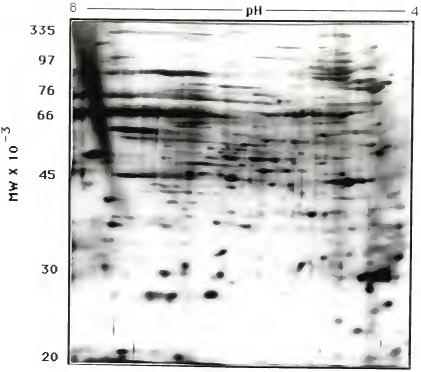
ANTI-ALBUMIN



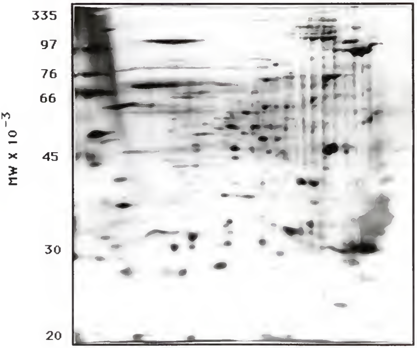
NRS

Figure 4-2. Silver stained 2D-SDS-PAgels of proteins secreted by FRLA-209-15 cells at the restrictive (40°C) and permissive (33°C) temperatures.

Cells were initially grown at the permissive temperature (33°C) for 3 days, extensively washed, and placed into serum free media. The cells were divided into two groups, one of which was transferred to the restrictive temperature (40°C) and the other (control) group maintained at 33°C . After an additional 2 days of incubation at their respective temperatures, conditioned media from the cells were collected and processed for analysis. Aliquots (100 µg) of the processed proteins from the two groups of cells were analysed by 2D-SDS-PAGE followed by silver staining as described in the "Methods" section. (Top panel) Silver-stained 2D-SDS-PAGE analysed proteins from cells grown at 40°C; (Bottom panel) Silver stained 2D-SDS-PAGE analysed proteins from cells grown at 33°C.



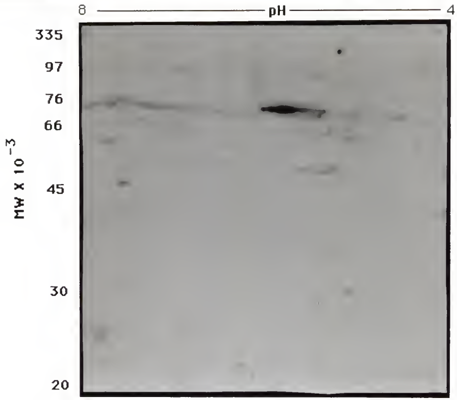
FRLA-209-15 at 40°



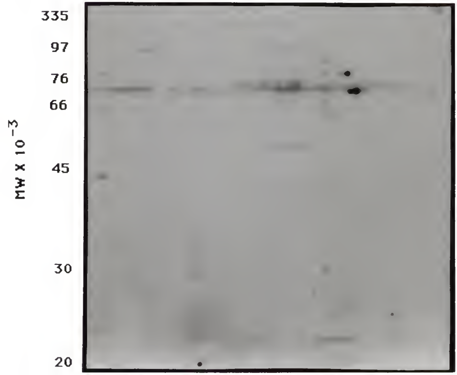
FRLA-209-15 at 33°

Figure 4-3. Immunoblot analysis of secreted proteins by FRLA-209-15 cells at the permissive (33°C) and restrictive (40°C0 temperatures.

Protein samples (100 μg) prepared from cells grown at both temperatures were subjected to 2D-SDS-PAGE under identical conditions as in Figure 4-2, transferred onto nitrocellulose, then stained with rabbit anti-rat albumin antibody, and immunodetected with [^{125}I]-labelled *Staph aureaus* protein A. (Top panel) Cells grown at the restrictive temperature (40°C); (Bottom panel) Cells grown at the permissive temperature of 33°C.



FRLA-209-15 at 40°
ANTI-ALBUMIN



FRLA-209-15 at 33°
ANTI-ALBUMIN

Figure 4-4. Immunostained 1D-SDS-PAGE showing the effects of temperature on proteins secreted by neonatal hepatocytes.

Primary hepatocytes initially grown at 37°C for 3 days in RPMI-1640 were extensively washed and placed in α-MEM-2. The plates were divided into 2 groups, one of which was kept at 37°C and the other at 33°C. After two additional days of incubation at these temperatures, the cells were extensively washed and the medium replaced with serum-free α-MEM-0. The cells were further divided into two groups each; one half of each group was transferred to 40°C while the other half remained at the original temperature, giving four groups in all. These were 33°C, 37°C, from 33°C to 40°C and from 37°C to 40°C. Conditioned media from these four groups were analysed by 1D-SDS-PAGE, electroblotted onto nitrocellulose, and stained with rabbit anti-rat transferrin antibody (Top), or pre-immune serum (Bottom). Protein from 2 x 10^6 cells was loaded on a gel. NRS means normal rabbit serum or pre-immune serum

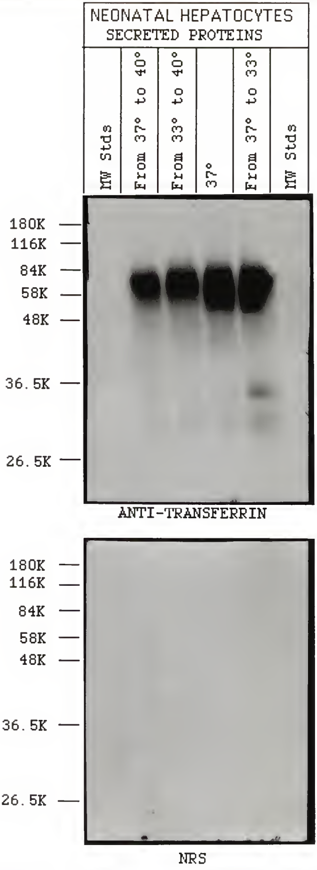

NEONATAL HEPATOCYTES
SECRETED PROTEINS
MW Stds
From 37° to 40°
From 33° to 40°
37°
From 37° to 33°
MW Stds
180K
116K
84K
58K
48K
36.5K
26.5K
ANTI-TRANSFERRIN
180K
116K
84K
58K
48K
36.5K
26.5K
NRS

TABLE 4-2

Effects of temperature on growth and viability of primary neonatal hepatocytes

Temperature	Cell number x (10^6)/dish	% viability
33°C	4.08	92
37°C	4.65	93
33°C->40°C	5.15	90
37°C->40°C	4.15	87

Cells were seeded at a density of 7 x 10^6 in 33 mm petri dishes and initially grown at 37°C for 3 days in RPMI-1640. The plates were divided into 2 groups, 37°C and 33°C. After two additional days of incubation at these temperatures, the cells were again extensively washed and the medium was replaced with serum-free α-MEM. The cells were again divided into two groups each: one half of each group was moved to 40°C and the other half remained at the original temperature, giving a total of four groups. That is 1) 33°C, 2) 37°C, 3) 33°C to 40°C, and 4) 37°C to 40°C. At the end of the incubation cells were trypsinized and counted with a hemocytometer. The viability was determined by the trypan blue exclusion method.

CHAPTER 5
THE β-ADRENERGIC RECEPTOR (βAR) AS A MARKER OF DIFFERENTIATION

Introduction

Retinoids induce or suppress differentiation in many cell types (Chou *et al.*, 1982). The human neuroblastoma cell line SK-N-BE can be induced to differentiate by either retinoic acid (RA) or α-difluoromethylornithine (DFMO). The RA induction causes a 60% reduction in growth rate, enhances γ-aminobutyric acid (GABA) and acetylcholinesterase levels, promotes neurite outgrowth, and caused overexpression of neural antigens (Melino *et al.*, 1988). In contrast, DFMO causes complete growth inhibition, cell body elongation, and higher binding of antibodies directed against neuroectodermal antigens, presumably because of enhanced expression of these antigens.

Chou (1977 and 1985) established temperature sensitive placental and fetal rat liver cell lines that can be induced to differentiate in response to temperature. These cells are temperature sensitive with respect to growth and the synthesis and secretion of tissue specific proteins. In addition, differentiation in their liver cell line is enhanced by retinoids and glucocorticoids (Chou *et al.*, 1988). However, there have been no reports of any accompanying molecular changes on the plasma membrane during these transitions, except that these cells retain functional glucagon receptors at both temperatures. Although there has been intense interest in the molecular changes at the cell surface that accompany cell transformation, current knowledge is still incomplete. The complexity of most plasma membranes, most of which at a minimum contain hundreds of different components, makes such studies inherently difficult. The techniques needed to critically compare the plasma membranes of normal and transformed cells are becoming available. Most attention has gone towards examination of the major outer plasma membrane proteins (Watson *et al.*, 1987). In these studies at least one change common to many types of cancer cells has been found: the reduction or loss of fibronectin (Hynes, 1973). Fibronectin is found as a fibrillar network surrounding cells in culture; the network disappears upon transformation of many cell types by RNA or DNA tumor viruses (Hynes *et al.*, 1982).

Kunos in 1968 reported a temperature- dependent shift from α- to β-adrenergic responses in isolated frog hearts. Recent observations have confirmed the existence of temperature-dependent inverse changes in α- and β-responses not only in the myocardium (Tirri *et al.*, 1984) but also in other tissues (Corwin *et al.*, 1982; Moreland *et al.*, 1984). More importantly, factors other than temperature induce apparent "conversions" of the adrenergic receptor responses (Kunos, 1984). The mechanisms for these apparent "conversions" are not clear but are believed to be linked at the level of G protein (Kunos, 1980).

Catecholamines have important metabolic effects in the liver: they stimulate glycogenolysis as well as gluconeogenesis and inhibit glycogen synthesis. In normal adult male rats, the glycogenolytic effect of catecholamines is mediated by α_1-adrenergic receptors, typically a cAMP-independent process. However, the same response is mediated predominantly by βARs in fetal (Sherline *et al.*, 1974) or female rats (Studer *et al.*, 1982) and in male rats under certain conditions. These conditions include thyroid (Preiksaitis *et al.*, 1979) and/or glucocorticoid (Chan *et al.*, 1978) deficiencies, malignant transformation (Christofferson *et al.*, 1975), liver regeneration (Garcia-Sainz *et al.*, 1986) or fasting (El-Refai *et al.*, 1982). A common denominator amongst most of these conditions is the lower level of cellular differentiation. This observation suggests that a change in the adrenergic receptor phenotype is part of the process of growth and differentiation (Kunos *et al.*, 1987). Since FRLA-209-15 cells are originally derived from fetal liver cells it is reasoned that either αARs or βARs mediate the metabolic effects of catecholamine.

This chapter describes experiments which explore some of the plasma membrane changes that accompany the temperature-induced differentiation of FRLA-209-15 cells. Screening for the expression of α- and β-ARs by the two phenotypic forms is a rational approach for the following reasons: a) catecholamines have important metabolic effects in the liver, and in the fetal liver this action is mediated predominantly through βARs; and b) Chou *et al.* (1985) have reported the retention of another liver specific function, namely the expression of the glucagon receptor, by FRLA-209-15 cells at both permissive and restrictive temperatures. The goal of these experiments is to develop a simple, readily measurable marker for the phenotypic changes in FRLA-209-15 cells. That being the case it is hoped that FRLA-209-15 cells can eventually provide a model system for the study of adrenergic receptor turnover using irreversible ligands. Such studies have been difficult in the past because it has been difficult getting a model system in which growth can virtually be arrested, as is the case in FRLA-209-15 cells. In these studies, it is also desirable to confirm that the cells expressed glucagon receptors as previously reported. The rationale for this experiment is based upon an observation by Dr. Kilberg of Dept. of Biochemistry, University of Florida (personal communication), that his laboratory is not able to demonstrate glucagon receptors in this line of FRLA-209-15 cells.

We also examined the influence of temperature on another function of plasma membrrane, the capacity to transport polyamines (as measured by transport of an analog).

Results

Adrenergic Receptor Expression by FRLA-209-15 Cells

The expression of adrenergic receptor types by FRLA-209-15 cells at the permissive and restrictive temperatures was studied by screening for the presence of α-and β-ARs as described in Chapter 2. Figure 5-1 shows the Scatchard analysis of ligand binding studies using [^{125}I]CYP. At 33°C, FRLA-209-15 cells expressed, per mg protein, βARs with a Kd of 32 pmol and a Bmax of 14 pmol/mg. At 40°C, there were 2-3 fold excess of βARs compared to 33°C, no change in Kd, and a Bmax of 30 pmol. In contrast, no αARs were expressed at either 33°C or 40°C.

Course of Differentiation

The time course of FRLA-209-15 cell differentiation as measured by differential expression of βARs was studied in FRLA-209-15 cells grown at the restrictive temperature of 40°C. βAR expression was followed for 7 days. As can be seen in table 5-1, βAR expression peaked after 48 hours at the restrictive temperature and remained at that level throughout the study period. It was concluded that differentiation of FRLA-209-15 cells at the restrictive temperature appeared to be completed by 48 hours. In all subsequent differentiation experiments FRLA-209-15 cells were grown for at least 48 hours at the restrictive temperature of 40°C. This finding supports the data on secreted proteins in Chapter 4.

Effects of Glucocorticoids (Betamethasone) on Differentiation of FRLA-209 Cells

The effects of betamethasone, a known differentiating agent and a potent glucocorticoid, on the βAR expression of FRLA-209-15 cells was studied. Betamethasone, 25 nM, was used in this experiment because the range of 5-30 nM is commonly reported for *in vitro* studies. Cells at the permissive (33°C) temperature were treated before, during, and after transfer to the restrictive (40°C) temperature to determine if the drug affected the amount of βARs expressed per mg protein of membranes in these cells. Table 5-2 depicts the amount of βAR expressed in the various treatment groups. Treatment with the steroid did not change the receptor number (Bmax) in any group, whether at the restrictive or permissive temperature. The determining factor was whether or not a cell was exposed to the restrictive temperature but not bethamethasone exposure. Efficiency of coupling or response, however, as measured by changes in the concentration and activity of cyclic AMP was not determined.

Membrane Transport Changes in FRLA-209 Cells

To determine whether the temperature-induced differentiation of these cells was accompanied by membrane transport changes, the ability of FRLA-209-33 and FRLA-209-40 cells to accumulate exogenous polyamines was studied by following [^{14}C]MGBG uptake for 24 hours. Figure 5-2 shows the ability of these two groups of cells to accumulate labelled [^{14}C]MGBG from the medium after 24 hours of incubation. There is an eight fold decrease in the drug accummulation capacity of the differentiated cell compared to the transformed cells.

Glucagon Stimulated Cyclase

To confirm that FRLA-209-15 cells expressed functional glucagon receptors, the ability of glucagon to stimulate cAMP production in 2 different groups of FRLA-209-15 cells was studied. Cells which were frozen at the time of receipt from the originator (new), as well as cells that have been in culture for sometime (old), were compared. Table 5-3 shows the results of a typical experiment. There was about a two fold increase in glucagon stimulated cAMP in the new stock cells (cells that had been frozen at the time of receipt from Dr. Chou), whereas there was no change in the old cells (cells that had been in culture for some time). Similar results were obtained when papaverine or rolipram were used as the phosphodiesterase (PDE) inhibitors.

Discussion

FRLA-209-15 cells express βARs at both the restrictive and the permissive temperatures. However, at 40°C, when the cells are more like hepatocytes, there is a 100-200% increase in the amount of βARs expressed (Figure 5-1). This finding suggests that the cells retain some of their hepatocyte characteristics even after transformation with SV40, as described for glucagon receptors by Chou *et al.*, (1985). The fact that no detectable α-receptors are seen suggests that transformation leads to the loss of some characteristics which do not reappear even at 40°C. The increased expression of βARs at 40°C can be used as an end point of differentiation in FRLA-209-15 cells.

The differential expression of βARs was used as an end point to follow the course of differentiation of FRLA-209-15 cells. The data suggest that the process of differentiation starts within 24 hours of transfer to the restrictive temperature (40°C), and that it is completed by 48 hours. The elevated receptor numbers remain at that level for the rest of the experimental period. On the basis of this experiment FRLA-209-15 cells were grown at 40°C for at least 48 hours before being regarded as differentiated. It must be pointed out that a similar time course is obtained by observation of the secretion of the liver specific proteins albumin and transferrin at 40°C (Figure 4-1). Chou *et al.* (1985 and 1988) have reported that FRLA-209-15 retains functional cAMP stimulatable glucagon receptors even after viral transformation. However, we and others cannot demonstrate the same extent of glucagon stimulated adenylate cyclase activity (Table 5-3). It may be that having been in culture for some time these cells have lost the glucagon receptors or the coupling to the second messenger systems. Even in stock cells that have been frozen at the time of receipt from Dr. Chou, glucagon stimulated adenylate cyclase activity cannot be demonstrated by us, which is in agreement with the observations made by Kilberg's group (personal communication). The stock glucagon used was initially dissolved in DMSO, then further diluted to the required concentrations with double distilled H_2O. The absence of a glucagon response prompted me to repeat the experiment using glucagon that was dissolved in a low pH solution (pH <3.5) as recommended by Kilberg (personal communication). The absence of a response in both cases makes the possibility of glucagon not going into solution or having been inactivated unlikely. PDE inhibitors other than IBMX were tried with similar results. This virtually eliminates the possibility of incomplete or differential PDE isoform inhibition. This finding is not unprecendented because Chou *et al.*, (1985) have reported that continuous culturing leads to the loss of certain cellular characteristics, including the ability to secrete liver specific proteins at 40°C; and our own observation of a decrease in the βAR expression with prolonged culture. Since glucagon stimulated cyclase activity could not be demonstrated, we were prompted to use another independent marker to confirm differentiation. The need for a second marker comes with the report of the βAR down regulation induced by DFMO (Dr Baker, Dept. of Pharmacology, Univ of Florida, personal communication). We are investigating the effects of DFMO on the phenotypic expression of FRLA-209-15 cells and are thus compelled to find a second marker.

Since agents like retinoic acid and glucocorticoids are known to induce differentiation in a number of cells both *in vitro* and *in vivo* (e.g. shrinkage of thymic cells in culture), the effect of betamethasone, a powerful glucocorticoid, on the βAR expression by FRLA-209 was studied. Our data suggest that betamethasone does not significantly affect the number of βARs per mg protein. However, we do not know whether betamethasone affects the efficiency of coupling of the βAR to its second messenger system. Chou *et al.* (1985), have reported that 10 nM dexamethasone increases the secretion of the liver specific proteins, albumin and transferrin, and inhibits the production of α-fetoproteins in FRLA-209-15 cells at 40°C. In addition, dexamethasone enhances glucagon stimulated cyclase but not receptor numbers.

As mentioned earlier, there have been reports suggesting that chemical or viral transformation of cells is often accompanied by surface modications of plasma membrane components. Some of the reported changes include changes in transport proteins for amino acids (Handlogten *et al.*, 1986), glucose and polyamines (Wallace *et al.*, 1982). The ability of FRLA-209-33 and FRLA-209-40 cells to accumulate exogenous polyamines was studied by following [^{14}C]MGBG uptake for 24 hours to determine whether the temperature-induced differentiation of these cells was accompanied by changes similar to those reported in other cells. There was about an eight fold decrease in the capacity of the differentiated cells to accumulate the drug compared to transformed cells; however, it remains to be determined what fraction of the observed difference is the result of differences in growth rate and efflux. Since MGBG is not metabolized (Warrel and Burchenal, 1983), it is not likely that differences in the rate of metabolism can account for the differences in the intracellular accumulation of MGBG.

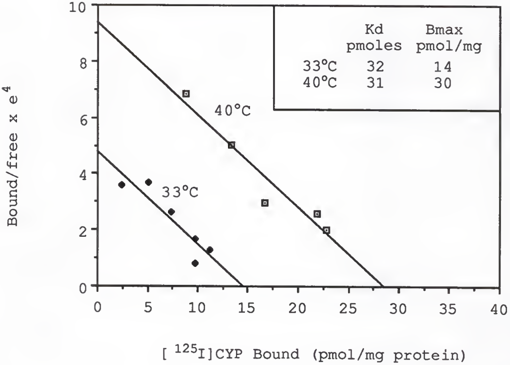


Figure 5-1. A typical Scatchard plot of specific [^{125}I] CYP binding to whole cell homogenates from FRLA-209-15 cells grown at the permissive (33°C) and the restrictive (40°C) temperatures.

Cells were grown initially at 33°C for 3 days then divided into two groups, 33°C and 40°C respectively. The two groups of cells were grown at the respective temperatures for an additional 48 hours. The cells were then washed extensively with PBS, detached from the plates, and whole cell homogenates were assayed for βAR with 6-100 pM [^{125}I]CYP as described in the "Methods" section. The data were plotted as the ratio of the amount of specifically bound ligand (pmol/mg protein) to free ligand (pmol/l) versus the amount of specifically bound ligand/mg protein. Data points are the mean of triplicate determinations.

TABLE 5-1

Effects of differentiation on expression of βARs.

Sample	fmol β-Adrenergic binding sites/ mg protein
33°C	15.0
40°C for $_{1d}$	22.0
2d	35.0
3d	32.0
4d	34.0
5d	32.0
6d	29.0

The numbers in subscript (1d) represent the number of days of growth at 40°C.

The time course of differentiation in FRLA-209-15 cells as monitored by the differential expression of βAR. Cells were grown at the permissive temperature (33°C) for 3 days (t=0) and then split into two groups. One group, the control (33°C), was harvested and processed for the receptor assay. The second group of cells was moved to the restrictive temperature (40°C) and grown for 6 more days. At 24 hour intervals cell samples were detached and processed for receptor assays as described in the "Methods" section. Each data point represents the mean of triplicate determinations and standard errors were less than 5% in all samples.

TABLE 5-2

Effects of betamethasone on βAR expression by FRLA-209-15.

Sample	fmol β-adrenergic Binding sites/mg protein
33°	11
33° + B_{2d}	12
40°	42
40° + B_{2d}	42

B_{2d} means exposure to Betamethasone for 2 days.

The effects of glucocorticoids on the expression of βAR by FRLA-209-15 cells grown at the permissive and restrictive temperatures. Cells initially grown at the permissive temperature (33°C) for 3 days were exposed to 25 nM betamethasone (B) before or during transfer to 40°C and then assayed for receptor expression as described in the "Methods" section. Each data point was the mean of triplicate determinations and standard errors were less than 5% for all treatments.

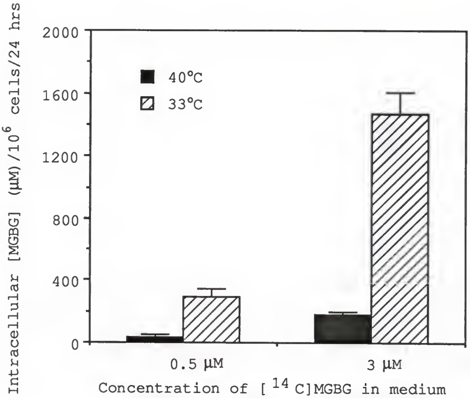


Figure 5-2. The accumulation of [^{14}C]MGBG into FRLA-209-15 cells grown at the permissive and restrictive temperatures.

Cells cultured at 33°C for 3 days (transformed) and cells transferred to 40°C for 48 hours (differentiated) were exposed to either 0.5 μM or 3.0 μM of [^{14}C]MGBG in the incubation medium at their respective temperatures. After 24 hours the cell-associated radioactivity was determined as described in the "Methods" section. Results are expressed as the mean ± SE of 3 determinations, each in duplicate.

TABLE 5-3

Effects of glucagon treatment on cAMP production by FRLA-209-15 cells.

Cell Type	Basal	Glucagon-stimulated
	pmol cAMP/10^6 cells/5 mins	
Old [a]	2.76 ± 0.2	3.88 ± 0.8
New [b]	3.41 ± 0.5	5.29 ± 1.3

[a] Represent FRLA-209-15 cells that had been frozen at time of receipt in 1986.

[b] Represent working stock of cells that had been in culture since 1986.

Attached FRLA-209-15 cells were incubated in HBSS containing 0.5 mM IBMX with and without 0.1 µM glucagon for 5 minutes at 33°C. At the end of incubation, the supernate was quickly replaced with ice-cold water and the cells were scraped and put immediately in boiling water for 5 minutes followed by centrifugation at 13,000 x g for 5 minutes. The supernates were assayed for cAMP content as described in the "Methods" section. Parallel plates were counted for cell number determination. Each point was the mean ± SE of 3 determinations, each in duplicate.

CHAPTER 6
THE ROLE OF POLYAMINES IN DIFFERENTIATION AND DE-DIFFERENTIATION OF FRLA-209-15 CELLS

Introduction

The polyamines, putrescine, spermidine and spermine, have been implicated in the regulation of cellular proliferation and differentiation (Pegg and McCann, 1982; Sunkara and Prakash, 1984). Inhibition of polyamine biosynthesis by DFMO results in the inhibition of tumor growth in a number of transplanted and chemically induced animal tumors (Sunkara *et al.*, 1987). Although the essential role of polyamines in tumor cell proliferation is well established, their role in the expression of a differentiated phenotype has yet to be clarified (Melino *et al.*, 1988). However, intracellular levels of polyamines fluctuate with cell differentiation (Pegg, 1986) suggesting their involvelment.

Whereas depletion of cellular polyamines by DFMO prevents the development of the differentiated phenotype in one group of cells (Erwin *et al.*, 1984; Kufe *et al.*, 1984), it stimulates other tumor cells to differentiate (Chen *et al.*, 1983; Kapyaho *et al.*, 1985). Moreover, addition of polyamines to the culture media reverses the DFMO effects in all cases, again suggesting the involvement of polyamines in cell differentiation. Attention has focussed in recent years on the involvement of polyamines in the replication of a number of human pathogenic DNA viruses. DNA viruses, including vaccinia and human cytomegalovirus, cause the activation of polyamine biosynthesis. This activation has been shown to be an absolute requirement for replication (Tyms *et al.*, 1982). Pretreatment with DFMO restricts the replication of herpes simplex virus and Semiliki forest virus. The fact that polyamines seem to be intimately associated with growth, differentiation, and the succesful establishment of transformation, induced either with chemical carcinogens or viruses, suggests that these organic polycations play important roles in these processes. However, whether polyamines are absolutely required remains to be determined.

As described earlier, FRLA-209-40 cells are temperature-induced, differentiated cells that can be made to de-differentiate by exposing them to 33°C for approximately 24-48 hours. This chapter deals with experiments aimed at defining the role of polyamines in the temperature-induced diffentiation and/or de-differentiation of FRLA-209-15 cells. In earlier studies (Chapter 3) it was demonstrated that the differentiation of FRLA-209-15 cells is accompanied by a greater than 50% loss of intracellular polyamines. Transfer of cells to the permissive temperature leads to re-acquisition of the transformed phenotype which is preceded by a repletion of the polyamine levels. This phenomena in effect involves re-establishing the SV40 virus infection. The effects of prevention of polyamine repletion may demonstrate the passive or directive role of polyamines in the process of transition between the two phenotypes. Briefly, the areas to be covered are: 1) the effects of DFMO on the growth and differentiation of FRLA-209 cells as measured by the secretion of the liver specific proteins transferrin and albumin; 2) the effects of spermidine and putrescine on the growth of these cells in order to determine the optimal dose necessary to block DFMO's effects on FRLA-209-15 cells; 3) the effect of DFMO on de-differentiation when FRLA-209 cells are moved from the restrictive to the permissive temperature; and 4) the possible reversibility of the effects of DFMO on de-differentiation by spermidine and other polyamine analogs such as bis-ethylspermine. Differentiation and de-differentiation were monitored by growth and by differential secretion of the liver-specific proteins, albumin and transferrin. The goal of these experiments was to test my hypothesis that polyamines play a directive role in the expression of the differentiated/transformed phenotype of FRLA-209-15 cells. In the long term, it is hoped that a model system can be developed that will enable the screening and classification of newly synthesized polyamine analogs as agonist or antagonist based on their ability to reverse DFMO's effects on differentiation and de-differentiation.

Methods

The methods were as described in Chapter 2. Sterile stock solutions of DFMO, putrescine, and spermidine were prepared in PBS and adjusted when necessary with 1 M NaOH to pH 7.5. Dilutions for individual experiments were prepared in PBS.

Results

Effects of Putrescine and Spermidine on Growth of FRLA-209-15 Cells

The effects of putrescine on growth and viability of FRLA-209-15 cells at the permissive temperature of 33°C is shown in table 6-1. Treatment of cells with 1 μM-1 mM putrescine for 6 days affected neither growth nor viability. Putrescine concentrations in the 100 μM to 600 μM range are the commonly used in reversal experiments; 500 μm was used in my reversal experiments. In contrast, exposure to similar concentrations of spermidine caused severe toxicities. After 24 hours of treatment, cells receiving higher concentrations (i.e. ≥100 μm) appeared malformed, rounded up, and detached. After 48 hours cells exposed to 30 μm spermidine began showing signs of toxicity and the cell count was about 95% that of control. Figure 6-1 shows the effects of spermidine (≤30 μM) on the growth of FRLA-209-15 cells. There was no observable inhibition after 4 days of treatment. After about 11 days of treatment, about 50% inhibition of growth was observed with 10 and 20 μM doses. Concentrations of 3 μM or less had no appreciable effect. Consequently 20 μM spermidine was used for reversal experiments, because this dose was not toxic to the cells after 48 hours treatment.

Effects of DFMO on the Phenotypic Expression of FRLA-209-15 Cells

Figure 6-2 shows the effects of DFMO on the transition from the differentiated to the transformed phenotype as detected by immunoblot analysis for transferrin expression. As described earlier (Figure 3-3), transfer of cells from 40°C to 33°C was associated with a repletion of polyamine levels which was then followed by a return to the transformed phenotype. As shown in Figure 6-2, transfer from 40°C to 33°C in the presence of DFMO delayed but did not prevent the return to the transformed phenotype as indicated by the decrease in transferrin expression. However, in the presence of DFMO growth inhibition was detectable by day 3. In the absence of DFMO the return was faster and almost complete in 24 hours (Figure 6-2; lane 4), whereas it took about 48 hours in the presence of DFMO (Figure 6-2; lanes 5, 6 and 7). Pretreating cells at 40°C with DFMO before the transfer also delayed the recovery (Figure 6-2 lanes 6 and 7). It was necessary to maintain continuous exposure to DFMO or the effect was lost within 24 hours (Figure 6-2; compare lanes 10 and 12 with lane 11). The presence of spermidine (20 μM) seemed to prevent the effect of DFMO (Figure 6-2; lanes 8 and 13). These facts have been represented graphically in Figure

6-3 which is a scanning laser densitometer quantitation of the immunoblot shown as Figure 6-2. Overall the effect of DFMO was marginal.

Discussion

This study has shown that DFMO treatment of FRLA-209-15 cells at 33°C is associated with growth inhibition unaccompanied by differentiation as would have been indicated by enhanced synthesis and secretion of the liver specific proteins transferrin and albumin. This conclusion is based on the fact that there is no increase in the secretion of albumin and transferrin.

These observations suggest that in the course of the temperature-induced differentiation in FRLA-209-15 cells, DFMO treatment and hence polyamine depletion does not play a determinant role. This is not entirely surprising because there are numerous reports of fluctuations in polyamine levels during cellular proliferation and differentiation. In some of these systems DFMO treatment either inhibited or enhanced differentiation. Consequently, it is neccessary to establish the importance of polyamine changes for each particular system. Since polyamines are capable of nonspecific interactions with cellular macromolecules, the need to distinguish specific from nonspecific interactions in a given system becomes even more important. Our studies have shown that the >50% reduction of putrescine and spermidine seen during differentiation and the elevation that precedes de-differentiation and growth upon transfer back to 33°C do not play a directive role. Inasmuch as the presence of DFMO during transfer from 40°C to 33°C delays the rate of return to the de-differentiated phenotype, we can conclude that the role of polyamines in our model system is modulatory. Prevention of polyamine re-accumulation does not prevent the the phenotypic transition from occurring, as would have been expected if they play a directive role. This observation makes the FRLA-209-15 model system not the ideal one to test our hypothesis that the newer polyamine analogs may have some agonistic functions. On the other hand, the system has allowed the separation of DFMO's effect on growth from its effect on differentiation. It also demonstrates that the increase in serum protein secretion by FRLA-209-15 cells at 40°C does not occur because the cells stopped growing. Had that been the case one would have expected similar increases when growth is inhibited with DFMO. It remains possible, of course, that more complete elimination of polyamines would change our conclusions.

DFMO enters the cell by simple diffussion (Tyms *et al.*, 1988), and the temperature-induced phenotypic changes are associated with membrane changes some of which were concerned with transport of amino acids and polyamines as described in Chapter 5. Thus, there is the possibility that these changes may affect the rate at which DFMO enters the cells, especially upon considering the fact that DFMO is essentially an amino acid.

Fetal hepatocytes can maintain their differentiated fetal state, mature to the adult state, or de-differentiate (Chou, 1988). The differentiated state of mammalian fetal liver can be monitored by the continued expression of liver specific genes. α-Fetoprotein (AFP), albumin, transferrin, and their respective mRNAs accumulate during early developmental stages, but a selective decrease in both AFP and AFP mRNA occurs after birth (Tilghman and Belayew, 1982). In contrast, production of albumin and transferrin increases after birth (Thomas and Schreiber, 1985). Thus, maturation of fetal hepatocytes can be monitored by a decrease in the expression of the AFP gene and an increase in expression of the albumin and transferrin genes. Differentiation can be measured by an increase in all three proteins under appropriate conditions.

TABLE 6-1

Effects of putrescine on growth and viability of FRLA-209-15 cells.

Putrescine (μM)	Cell number [a] ($x10^6$)	Viability [a] (%)
Control	5.8	>90
3	6.1	>90
30	6.2	>90
100	5.8	>90
300	6.0	>90
1000	6.2	>90

[a] Results are from representative experiments.

Cells were grown at 33°C in α-MEM-2 with medium changes every other day. Putrescine was added 24 hours after seeding and was maintained in the medium at each medium change or passage. Cells were counted with a coulter counter (model ZF) and cross checked with a haemocytometer. Viability was determined by the trypan blue exclusion method. Cells were exposed to the indicated concentration of putrescine for 6 days.

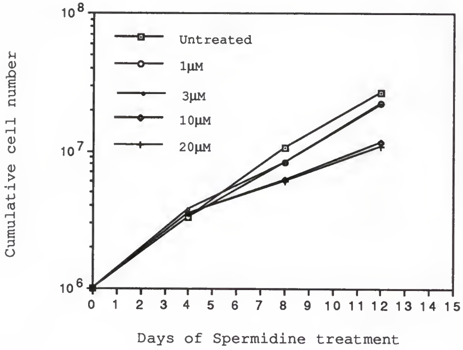
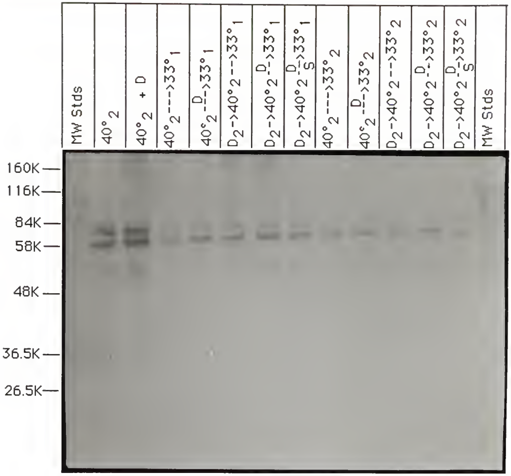


Figure 6-1. Effects of Spermidine on the growth of FRLA-209-15 cells at 33°C.

Cells were grown at 33°C in α-MEM-2 with medium changes every other day. Spermidine was added 24 hours after seeding and the spermidine was maintained in the medium at each medium change or passage. Cells were counted with a coulter counter (model ZF) and cross checked with a haemocytometer. Viability was determined by the trypan blue exclusion method. Cells were exposed to the various concentrations of spermidine for 6 days.

Figure 6-2. Immunostained 1D-SDS-PAgels showing the effects of DFMO on the transition of FRLA-209-15 cells from 40°C to 33°C.

Cells initially grown at 33°C for 3 days were transferred to 40°C with and without DFMO for 2 days. After the incubation the cells were again transferred to 33°C with and without DFMO and spermidine as indicated on the blot. Media and DFMO was replaced everyday. Samples of the conditioned media were collected and processed for analyses by 1D-SDS-PAGE, followed by immunostaining with rabbit anti-rat transferrin (Top Panel) or pre-immune serum (Bottm panel), then immunodetected with [^{125}I] labelled protein A as described in the "Methods" section.
D = DFMO, S = Spermidine, 40_2 = cells at 40°C for 2 days, D_1 = DFMO treatment for 1 day.



ANTI-TRANSFERRIN

NRS

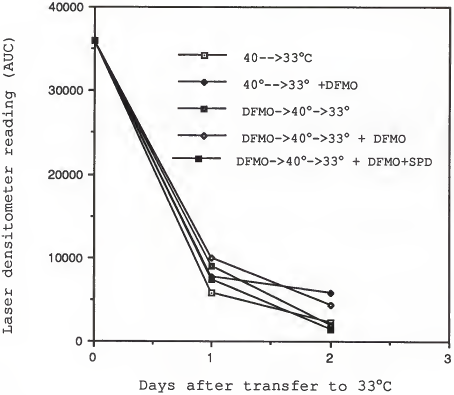


Figure 6-3. Laser densitometer quantitation of the anti-transferrin immunoreactive blot in fig 6-2 showing the effects of DFMO on de-differentiation.

1D-PAgels were run as described in Figure 6-2. The blots were quantitated on a Zeineh soft laser scanning densitometer. AUC represents area under the curve.

CHAPTER 7
SUMMARY AND CONCLUSIONS

Regulation of cellular proliferation is a complex process that involves both positively and negatively acting signals. Tumorogenesis likely results from alterations in genes whose protein products are involved in these signalling pathways. The DNA tumor viruses encode a set of proteins that are capable of overriding and reprogramming normal regulation of cellular growth; consequently, they have been widely used as model systems for studying cellular transformation. The oncogenes (tumor-inducing genes) from polyomavirus, SV40, and adenovirus are able to induce a number of distinct changes in cell phenotype, including immortalization, secretion of growth factors and other proteins, loss of contact inhibition, anchorage-independent growth, and morphological transformation (Whyte *et al.*, 1988).

FRLA-209-15 cells are fetal liver hepatocytes that have been transformed with ts mutants of SV40 virus. At the permissive temperature (33°C), the oncogene product 'T' is active and capable of overriding and reprogramming normal regulation of cellular growth. However, at the restrictive temperature (40°C) the gene product of the mutant virus is inactivated, and there essentially is a return to normal regulation of cellular growth. In the absence of an active virus at 40°C, FRLA-209-15 cells can differentiate to the original fetal hepatocyte state from which they were derived or mature to become adult hepatocytes (Chou *et al.*, 1988). The differentiated fetal hepatocyte is characterized by secretion of high levels of the fetal liver specific protein α-fetoprotein, moderate amounts of albumin and transferrin, and very little of the adult liver specific proteins tyrosine amino transferase (TAT) and acid glycoprotein (AGP). In contrast, mature hepatocytes secrete little or no AFP but secrete high levels of albumin, transferrin, and the maturation-specific (adult) proteins TAT and AGP. Consequently, monitoring the disappearance of AFP secretion is a mark of fetal cell differentiation, whereas the appearance of TAT and AGP marks maturation. Increases in albumin and transferrin, however, serve as a mark of differentiation in both fetal and adult hepatocyes (Kelly and Darlington, 1989; Thomas *et al.*, 1990; Woodworth *et al.*, 1988). The exact mechanism of the T protein-mediated override in FRLA-209-15 cells is unknown, but it is believed that "T" forms a complex with a number of anti-oncogenes which normally exert control on growth. Following the removal of the normal controls, the cells assume the transformed phenotype. Studies in other cell systems have identified the retinoblastoma gene product (RB), p53 and other cellular proteins like pp60 c-src as some of the host-cell-proto-oncogenes that T antigen complexes with. This complex formation has been presumed to be the mechanism in FRLA-209-

15 cells. In these cases, the formation of the complex has a profound effect on protein activity and stability.

The present study has characterized the regulation of cellular proliferation and differentiation in FRLA-209-15 cells at the permissive and restrictive temperatures with respect to a) the differential secretion of the liver specific proteins, albumin and transferrin; b) plasma membrane alterations that accompany these processes by differential expression of βARs; and c) fluctuations in polyamine levels and their impact on the expression of the differentiated and transformed phenotype, as well as cell cycle traverse. The study shows that growth, protein secretion, plasma membrane alterations, and intracellular polyamine levels are all temperature dependent. FRLA-209-15 cells are temperature sensitive and the change from one phenotype to the other is associated with fluctuations in intracellular polyamine levels.

To answer the question of polyamine involvement in differentiation and transformation as outlined above, it is necessary to have a reliable readily-measurable end points. I therefore characterized the temperature-induced changes in the secretion of albumin and transferrin to determine how it could best be exploited. As an alternative endpoint, FRLA-209-15 cells were also characterized with respect to the surface membrane alterations associated with the temperature-induced phenotypic alterations, specifically by following the differential expression of βARs. Based on these two approaches, it is apparent that, irrespective of the approach used, differentiation is completed by 48 hours.

To address the question of the role of polyamines in differentiation, the effect of DFMO treatment on transferrin secretion by FRLA-209-15 cells at 33°C, 40°C, and at time of transfer from 40°C to 33°C, was studied. Polyamine involvement in numerous cellular processes has been extensively documented in the literature (Pegg, 1986; Heby, 1981; Tabor and Tabor, 1984). Polyamines have been shown to be involved in growth, differentiation, cell cycle traverse, and transformation. Two contrasting views have been presented with respect to the role of polyamines in cell cycle traverse. One view holds that polyamines are important for a cell to progress through the cell cycle. In support of this theory, DFMO treatment, which results in the depletion of polyamines, causes transformed cells to arrest in S phase while non-transformed cells are arrested in G_1 (Prakash *et al.*, 1983; Sunkara *et al.*, 1981). However, there is a second school of thought that contests this conclusion, and these workers have reported that tumor cells arrest in G_1 (Oredsson *et al.*, 1983;) or G_2 (Anehus *et al.*, 1984; Harada *et al.*, 1981) after DFMO treatment. In my studies, FRLA-209-15 cells exposed to DFMO arrest in G_1. This finding is in agreement with the second view. However, it is evident from my studies and those of other workers that DFMO treatment affects cell cycle traversal differently in different cell systems. Consequently, generalizations regarding polyamine involvement in cell cycle traversal based on the use of DFMO as a probe should be made with caution.

Studies involving mutants defective in the polyamine biosynthetic pathway, as well as with use of DFMO, in various cell systems have also demonstrated the involvement of polyamines in transformation and differentiation (Tabor and Tabor, 1984). Whereas in some systems depletion of polyamines with DFMO prevents the development of the differentiated phenotype, it stimulates various tumor cell lines to differentiate (Melino *et al.*, 1988), a finding that suggests that the involvement of polyamines in cell differentiation is complex. However, the interrelationships between cellular proliferation and differentiation are not clear. Differences in the extent of polyamine depletion achieved, in addition to the fact that in some cases differentiation requires a critical number of cell replications (Suguira *et al.*, 1984), may account for some of these reported differences. These studies are conducted in tumor or normal cells stimulated to grow with hormones, growth factors, or serum. In these systems it is impossible to investigate the involvement of polyamines in both differentiation and proliferation in the same cells, because these cells express one phenotype or the other, but not both. However, the temperature sensitive FRLA-209-15 cell system provided just such an opportunity. In agreement with published reports, DFMO treatment causes inhibition of FRLA-209-15 cell proliferation at 33°C. However, DFMO has no significant effect at 40°C, once the cells are fully differentiated. Despite the fact that the transfer of FRLA-209-15 cells from 40°C to 33°C involves in effect the re-initiation of the action of T antigen, as well as the elevation of polyamine levels, DFMO treatment has only moderate effects on the re-emergence of the transformed phenotpye as measured by the loss of secreted transferrin. This observation is in conflict with published reports which indicate that the depletion of polyamines or the abolition of the elevation in polyamine levels that usually precedes growth or viral transformation is sufficient to abort these processes (Pegg, 1988). I conclude that in the FRLA-209-15 system, polyamines play only a modulating role in the transition. However, this study has clearly distinquished the growth promoting functions of polyamines from the effect on differentiation. The limited effect of DFMO on the re-emergence of the transformed phenotype could also be due to decreased uptake of DFMO at 40°C. The role of polyamines are unambiquous with respect to cell growth, but their role in differentiation and cell cycle traversal remains controversial.

Comparison of temperature-induced growth-inhibited cells with DFMO treated cells reveals some interesting differences and sheds light on the importance of the associated polyamine changes. Like increased temperature, DFMO inhibits the growth of FRLA-209-15 cells. However, whereas temperature effects on growth occurr within 24 hours, that of DFMO are gradual with a lag period of 4 days. Flow cytometer analysis of DFMO treated cells shows that cells become arrested in the G_1 phase. In addition, both temperature and DFMO treatment of FRLA-209-15 cells led to the depletion of polyamine levels. The extent of depletion due to DFMO is greater than that associated with the temperature-induced transition from the transformed to the differentiated phenotype. This observation is in agreement with the contention that the extent of depletion of polyamines accounts for the reported differences in the expression of the differentiated phenotype. Differentiation is associated with about a 50% decrease in both putrescine and spermidine, whereas there is a 90% reduction of these polyamines after treatment with DFMO for 6 days. These findings suggest that the growth inhibition observed under these two conditions may be caused by different mechanisms. In the DFMO treated cells the presence of a lag period before growth inhibition is consistent with the mechanism described for DFMO (Bey *et al.*, 1987). Since DFMO is a 'suicide' inhibitor that must be reacted upon to be converted to the reactive form (Bey *et al.*, 1987), time is required to inactivate the ODC enzyme. As no or very little polyamines are synthesized and the cells continue to divide the cells are depleted of polyamines and their growth become inhibited. However, the 48 hours required to complete the polyamine depletion cannot account for the immediate cessation of growth in the temperature-arrested cells, unless mechanisms other than polyamine depletion are involved. On the other hand, the pattern of growth inhibition due to temperature is consistent with the oncogene inactivation paradigm that has been proposed to explain the behaviour of FRLA-209-15 cells (Chou *et al.*, 1981; Decaprio *et al.*, 1988; Weinberg, 1988).

My study also confirms that at 40°C, the temperature-specific increase in protein secretion by FRLA-209-15 cells involves new protein synthesis, including that of the liver-specific proteins transferrin and albumin. Differentiation at 40°C is completed by 48 hours, and transfer back to 33°C is accompanied by reversal to the transformed phenotype. The emergence of the transformed phenotype is characterized by decreased protein secretion and loss of albumin and transferrin expression, both of which are completed by 48 hours. Immunoblot analysis revealed that the temperature-induced protein secretion by FRLA-209-15 cells is specific to these SV40-transformed fetal hepatocytes as the exposure of primary cultures of neonatal hepatocytes to 40°C results in a decrease in secreted proteins compared to cells at 33°C and 37°C. The temperature-induced reversible increase in the secreted liver-specific proteins, albumin and transferrin, observed in the FRLA-209-15 cell system is in agreement with the published data (Chou *et al.*, 1985). Moreover, my study has expanded on the utility of FRLA-209-15 cells as an investigative tool by defining the duration of differentiation and de-differentiation. This finding was exploited to demonstrate that even though there was a change in polyamine levels in FRLA-209-15 cells during phenotypic transitions, their role was modulatory as opposed to directive.

Furthermore, the amount of protein secreted by 2 X 10^6 neonatal hepatocytes is greater than that of an equivalent number of FRLA-209-15 cells. This conclusion is based on the fact that the blots of primary hepatocytes require only 3 hours of exposure to the X-ray films for sufficient signal development in contrast to 24-48 hours for FRLA-209-15 cells, even though the same lot and amount of iodinated protein A (corrected for decay) is used. One explanation for this difference in protein secretion is that perhaps the neonatal cultures have undergone the phenotypic transition from the fetal to adult hepatocyte that occurs at birth during normal development. At birth, there is a remarkable transition from the fetal to adult hepatocyte phenotype which is characterized by increased secretions of albumin and transferrin and diminished production of α-fetoprotein. This pattern is in contrast to the pattern of protein secretion observed in differentiated FRLA-209-15 cells discussed above. When fetal hepatocytes differentiate there is increased production of albumin, transferrin, and α-fetoprotein. Another explanation of the apparent quantitative difference between the primary cultures and FRLA-209-15 cells is that the FRLA-209-15 cells have been in culture for some time and according to various reports (Chou, 1981; Kelly and Darlington, 1989; Woodworth *et al.*, 1988), levels of expression of liver-specific genes change with time in culture.

One of the goals of my study was to define the time course of differentiation in FRLA-209-15 cells. This was achieved by monitoring the accompanying differential secretion of liver specific proteins and plasma membrane changes over time. The differential expression of βARs represents accompanying surface changes (Hynes, 1973; Kunos *et al.*, 1987). Interestingly, these two markers show that differentiation is complete within 48 hours of incubation at 40°C. That these two different approaches give similar results is very reassuring.

Comparing the expression of transferrin in DFMO treated and untreated cells indicates only a modulatory role for polyamines upon transfer from 40°C to 33°C. This finding is significant because it suggests that perhaps FRLA-209-15 cells may not be the ideal system to screen polyamine analogs for either antagonist or agonist activity, a long term goal of this study. Agonist activity is defined as the ability to enhance cell proliferation or to enhance the secretion of the liver-specific proteins transferrin and albumin. The use of the other end points of differentiation described previously are unsuitable for this series of experiments for several reasons. Firstly, DFMO treatment induces βAR down regulation (Dr. S. P. Baker, Dept. of Pharmacology, University of Florida, personal communication). Secondly, albumin secretion follows a biphasic pattern and is also dependent on cell density. In contrast, transferrin secretion is monophasic and independent of prior cell density (Chou, 1981). Consequently, since transferrin secretion has far less variables, it can be exploited as the end point for FRLA-209-15 cell differentiation.

In conclusion, these studies have characterized FRLA-209-15 cells with respect to a course of differentiation to de-differentiation. Furthermore, evidence has been provided to demonstrate that polyamines play a modulatory rather than directive role in the differentiation of FRLA-209-15 cells. The characterization of plasma membrane changes, particularly the differential expression of βARs, has also provided a useful model system for individuals interested in β-receptor turnover. This area has traditionally been a difficult undertaking in typical *in vitro* model systems because of the complicating factor of cellular growth. This difficulty is overcome in the FRLA-209-15 system where at 40°C adrenergic receptors increase and cellular growth ceases.

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BIOGRAPHICAL SKETCH

Daniel Yaw Danso was born in Accra, Ghana. He attended Saint Peter's Secondary School where he obtained his School and Higher School Certificates in 1976 and 1978, respectively. In January, 1979 he matriculated at the University of Ghana Medical School and graduated in July, 1985, with the MB.ChB degree. Thereafter, he did a year of internship at the University Hospital, spending six months in pediatrics and six months in surgery. While at the pediatric department he worked with the pediatric oncolgy unit, and his duties included administration of chemotherapy to children with Burkitts Lymphoma. He marvelled at the variations in response and toxicities shown by his patients and was encouraged to go to graduate school to learn more about chemotherapy in general. In the Spring of 1987, he enrolled in the graduate program of the the Department of Pharmacology and Therapeutics at the University of Florida. He began work toward a Ph.D. under the guidance of Dr. Allen H. Neims and later under the coguidance of Drs. Kathleen T. Shiverick and Thomas C. Rowe. After completing the requirements for the Ph.D., he plans to do a residency training in internal medicine at the St. Lukes Roosevelt Hospital Center in New York City.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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